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Cellular immunity of coronavirus-infected bovine from ecologically different districts of Lithuania

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The haematological and cellular immune biomarkers of healthy and coronavirus-infected bovine organisms in ecologically different districts of Lithuania were investigated. Serological screening of bovine blood serum against coronavirus revealed 12.1% of positive cows in the conventionally ecologically clearer Ukmergë district (<1 t/km^2/year of total contaminants) and conventionally ecologically contaminated Trakai district (60–62 t/km^2/year of total contaminants). The levels of erythrocytes, hemoglobin, total leukocytes and segmented neutrophils were decreased, while eosinophil and methemoglobin levels were increased in the blood of healthy and coronavirus-infected cows in the conventionally ecologically contaminated district. The immunoregulatory index (CD4 and CD8 cell ratio) was decreased more significantly (1.5 times) in coronavirus-infected cows in Trakai district than in Ukmergë district. Our results indicate that unfavourable ecological conditions influence the redistribation of immunocompetent cell populations, the development of immunodeficiency and the spread of viral infections in the organism.

Key words: contamination, coronavirus, lymphocyte population

Abbreviations: BCV, bovine coronavirus; FITC, fluorescein isothiocyanate; PBM, peripheral blood mononucleares; PBS, phosphate buffer; RNA, ribonucleic acid

INTRODUCTION

The immune system consists of a complex network of cells and molecules scattered throughout the body of all multicellular organisms and is able to recognize and neutralize potentially harmful agents, conferring to the organism resistance to infectious and malignant diseases. Optimal functioning of the immune system requires that specific cells and cell products intersect with each other in a sequential, highly regulated manner. Disruption of this regulation may alter the overall efficiency of the system, resulting in viral pathology.

Coronaviruses constitute a large genus of spherical or pleiomorphic enveloped particles and belong to the *Coronaviridae* family. Several of these viruses infect domestic animals and cause significant morbidity and mortality leading to major economic losses. Coronaviruses are widespread pathogens among mammalian and avian species and cause a number of important diseases that range from respiratory infections and gastrointestinal enteritis to hepatitis, encephalomyelitis, vasculitis and coagulopathies.

Bovine coronavirus (BCV), as an enteric coronavirus, causes acute enteritis in neuborn calves and chronic infections in adult cattle. As an enteropathogen, BCV causes severe diarrhea in neonatal calves, and it is also considered to be etiologically involved with winter dysentery of adult cattle (Donohni et al., 1976; Mebus et al., 1993; Saif et al., 1988). BCV possesses a complicated antigen spectrum in itself, which is defined by a specific protein amount. Various environmental chemicals can adversely affect the immune system and immune response. The immune system is one of the organ systems most sensitive to chronic low-dose exposure to chemical toxicants (Kilburn, Warshaw, 1994). An increasing number of chemicals have been manufactured every year and continue to be released in the environment. Among them, many chemicals have been shown to be immonologically toxic. Such chemicals may modify or disturb antigen processing, resulting in presentation of epitopes for which tolerance has not been established (Valentovic et al., 1997; Griem et al., 1998). Disturbances of the immune system due to toxicants may cause hyperreactivity as in allergy and autoimmunity or immunosupression that leads to infection-induced diseases (Lochmiller, Dabber, 1993; Mebus et al., 1993). Chemical and biological pollutants may affect the immune system locally or systemically, through antigen-dependent and antigen-independent activation pathways of the immune response. Determination of various lymphocyte types and concentration of immunoglobulin classes in the peripheral circulation gives a relevant indication of the cellular and humoral immune responses of a person or population.

Virus strain and dosage, environmental factors and the involvement of other secondary pathogens (opportunistic infections) as well as corpuscular antigens, viruses are sufficiently immunogenic and cause the immune response in cellular and humoral (CD4, CD8 lymphocytes, natural killer and other subpopulations of cells) immunity chains. The cellular immune system CD4 T cell subsets play the central role in the regulation of immune responses. It is well known that CD4 and CD8 molecules play a critical role in T-cell responses to antigen. The CD8 T lymphocytes are the main cellular effectors mediating elimination of virally infected or tumor antigen (Ag) and alloantigen-expressing cells (Auphan-Anezin et al., 2003). Immune function in animals can be adversely affected by long-term exposure to environmental contaminants. Investigating alterations in immunity can therefore yield a relevant information about the relationship between exposure to environmental contaminants and susceptibility to infectious diseases (Hansen, 2003). Evidently important would be to assess whether there is any relationship between exposure to pollutants and immune system biomarkers in healthy and coronavirus-infected animals.

The aim of our studies included the investigation of the influence of harmful exogenic factors on the cellular immune biomarkers of healthy and coronavirus-infected cows held in ecologically different districts.

MATERIALS AND METHODS

Districts and animals. Blood and serum samples of cows from herds were collected in Trakai (conventionally ecologically contaminated) and Ukmergë (conventionally ecologically clear) districts. For complex investigations blood samples were withdrawn from jugular veins of Ayashire and Lithuanian red breed cows 3–6 years old. The bovine were kept in herd complexes under similar feeding and veterinary control conditions.

Coronavirus infection detection. Overall 377 bovine were tested for coronaviral infection. Coronavirus infection was detected in blood serum samples with immunodiagnostic (immunodiffusion method) kits obtained at J. Kovalenko Experimental Veterinary Institute (Moscow, Russia) and Bio-X coronavirus ELISA Kit (Brussels, Belgium) according to manufacturer's instructions.

Mononuclear cell isolation. Peripheral blood mononuclear (PBM) cells were isolated by centrifugation on a Ficoll-Pague density gradient (1.077 g/m^3). The cells collected from the interphase were washed three times with cold phosphate buffer (PBS) and resuspended in RPMI-1640 medium.

Hematological and immune biomarker analysis. The hematological and immune biomarkers such as the total number of erythrocytes and leukocytes, hemoglobin and methemoglobin levels, leukocyte formula, T population, immunoregulatory index were studied by routine methods (Immunologijos…, 1997).

Flow cytometry analysis. Isolated PBM (5×10^5) were incubated in 50 µL PBS with fluorescein isothiocyanate (FITC) labeled antibodies. PBM labeling procedures with specific monoclonal antibodies against CD4, CD8 cells (supplied by Dr J. Naessens from ILRAD, Nairobi, Kenya) and resolution-positive cells by goat FITC-conjugated antimouse IgG polyclonal antibodies (Sigma, USA) were done as described (Larsen et al., 1990). The immunophenotyping of T lymphocyte CD4 and CD8 subpopulations was performed on a FACSort instrument (Becton Dickinson). FITC was registered through a 530/30 nm bandpass.

Data analysis. All data were expressed as mean ± SD values. The statistical significance was calculated using Student's unpaired t test, data were compared among different groups of animals.

RESULTS AND DISCUSSION

We have investigated the bovine serological, haematological and cellular immune parameters in conventionally ecologically clearer Ukmergë district (<1 t/km^2/year of total contaminants) and conventionally ecologically contaminated Trakai district (60–62 t/km^2/year of total contaminants). The contamination of the environment with benzo(a)pyrene (Ecological…, 1997) and nitrates was also more significant in Trakai district (Table 1).

Serological screening of bovine blood serum against coronavirus infection showed that the bovi-

Table 1. **Concentration of nitrate in ecologically different districts**

Indices	Concentration of nitrate		
	Ukmergë		Trakai
	mg/kg		
Grass	480 ± 20		770 ± 25
Hay	820 ± 40		1345 ± 40
Root	746 ± 35		1560 ± 34
	mg/l		
Water	35.0 ± 2.7		84.0 ± 3.5
Bovine:			
milk	10.5 ± 1.3		30.2 ± 2.6
blood serum	37.5 ± 2.7		75.8 ± 5.6

ne with positive reaction didn't comprise substantial numbers, namely 12.1% in Ukmergë and 15.9% in Trakai districts. One can see the that percentage of coronavirus-positive cows in the conventionally contaminated Trakai district was 1.3 times higher ($P < 0.01$) than in the conventionally clearer Ukmergë district. The reason for this phenomenon may be tightly linked with the disturbed intestinal (mucosal) immunoprotective features of cows in the contaminated district. The mucosal immune system is structurally and functionally distinct and specific for the interaction between the organism and its environment (Feng et al., 1999). Our results are compatible with the literature data in the part that through the ecological conditions people and animals become infected with corona-, rota-, diarrhoea and other viruses (Storz et al., 1991; Lochmiller, Dabber, 1993).

Typical coronaviruses are pleiomorphic to rounded particles 60–220 nm in diameter with club-shaped surface projections 12–24 nm in length, with a lipid envelope surrounded by a fringe of surface projections termed spikes or peplomers. Epithelial cells are important target cells for coronavirus infection. Coronaviruses replicate in the cytoplasm and assemble, bud at membranes of the intermediate compartament located between the endoplasmic reticulum and the Golgi complex. Like other enveloped viruses, coronavirus assembly is presumably dependent on protein localization and protein–protein as well as protein–RNA interactions (Risco et al., 1998).

BCV represents one of the better characterized coronaviruses according to hemagglutinin properties. Four major structural proteins are associated with infectious BCV. Hemagglutinin as well as the integral membrane glycoprotein M (23–26 kDa) are associated with the viral envelope while the phosphorylated N protein (50–54 kDa) functions as a nucleocapsid. Proteolytic cleavage of the S glycoprotein precursor into S1 and S2 of 100 and 110 kDa is required for cell fusion activity. The S1/S2 glycoproteins facilitate virus attachment to susceptible cells, and also binding to erythrocytes, cell fusion, and induction of neutralizing antibodies. These antibodies have been detected to persist in a positive group of cows during our serological screening. The exact functions of hemagglutinin and S1/S2 and their interplay in infections *in vitro* and *in vivo* are not yet fully elucidated (Schultze et al., 1991; St. Cyr-Coats et al., 1988; Storz et al., 1983; Storz et al., 1981).

Hematological investigations showed that in the conventionally contaminated Trakai district the levels of erythrocytes, hemoglobin, leukocytes and segmented neutrophiles were decreased, while those of eosinophil and methemoglobin (2–4% by total hemoglobin) increased in the blood of healthy bovine and in coronavirus-infected cows (Table 1, $P < 0.05$) than those in the conventionally clearer Ukmergë district. A comparison of the biomarkers revealed increased erythrocyte and segmented neutrophil levels, as well as a decrease in eosinophil count ($P < 0.05$) among coronavirus-infected bovines in the contaminated Trakai district. A decrease of eosinophil count was recorded among infected bovines ($P < 0.05$) in both Ukmergë and Trakai districts. The level of blood serum nitrates in healthy and infected cows was also markedly increased ($P < 0.05$) in the Trakai district (Table 1).

It is well known that the high level of nitrates in the foliage and water is the main reason for their reduction to increased concentracions of nitrites. Nitrites in the blood oxidize hemoglobin to the Fe^{3+} form. It may be supposed that the process of intoxication and the weak supply of oxygen to the tissues negatively influences the immunity of the organism and evokes allergization reactions (higher eosinophil counts). Prolonged accumulation of nitrates and their metabolites may disturb and weaken the immunity of the organism and cause the susceptibility to viral infections (Harrison, Jollow, 1987; Tamoðiûnas et al., 1998). Chemical pollution, one of the stress factors, is considered to be responsible for physiological alterations, as changes in pyruvate and lactate concentrations in affected cows have been determined previously (Weltzien et al., 1996).

Our investigations of bovine peripheral blood lymphocytes and their populations (CD4 and CD8 cells) have demonstrated the tendency of increment of the amount of CD8 lymphocytes both in healthy and coronavirus-infected cows in conventionally ecologically contaminated Trakai district (Table 2, $P < 0.05$). The immunoregulatory index (the CD4/CD8 cell ratio) was 1.3 times lower in coronavirus-infected cows in the conventionally contaminated Trakai district ($P < 0.05$) as compared to conventionally clearer Ukmergë district. The tendency of increment in CD8 (suppressor/cytotoxic) cell proportion possibly demonstrate not only the known fact of their involvement in the clearance of virus and infected cells, but also a shift of immunoregulatory functions when the organism is affected by unfavourable ecological conditions. Viral infections, as we have determined (Dringelienë et al., 2002), are related to some alterations in the cellular and/or humoral immunity chain interaction in the organism.

The coronavirus S, N and M-proteins are relevant targets for recognition by cellular immune (Enjuanes, 1995). N-protein is frequently involved in T-cell responses to coronaviruses (Bergman et al., 1993; Stohlman et al., 1995; Stohlman et al., 1992). Collaboration between B and T-cell antigenic deter-

Table 2. **The haematological biomarkers of healthy and coronavirus-infected bovine from different ecological districts**

Biomarker	District			
	Ukmergë		Trakai	
	I	II	I	II
Erythrocytes (10^{12}/l)	6.4 ± 0.4	6.8 ± 0.4	4.8 ± 0.3[1]	6.3 ± 0.5[2]
Hemoglobin (g%)	11.7 ± 0.9	11.0 ± 0.9	9.6 ± 0.4[1]	9.7 ± 0.2[2]
Methemoglobin	0–1% Hb	0–1%Hb	2–4% Hb[1]	1–3% Hb[3]
Leukocytes (10^9/l)	6.6 ± 0.3	6.4 ± 0.3	4.8 ± 0.8[1]	6.3 ± 0.3
Leukocyte formula (%)				
Neutrophils:				
Stick	4.3 ± 0.2	4.6 ± 0.2	3.7 ± 0.2	5.1 ± 0.3
Segmented	25.3 ± 0.7	25.4 ± 0.4	23.5 ± 0.7[1]	25.2 ± 0.5[2]
Eosinophils	6.2 ± 0.4	4.0 ± 0.3[2]	11.1 ± 0.5[1]	4.8 ± 0.4[2]
Basophils	0.7 ± 0.1	0.8 ± 0.2	1.2 ± 0.2	1.2 ± 0.2
Lymphocytes	60.5 ± 1.6	62.1 ± 1.4	56.1 ± 1.8	60.2 ± 1.2
Monocytes	2.5 ± 0.3	2.7 ± 0.4	2.8 ± 0.2	2.8 ± 0.2
Other cells	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1

I – healthy, n = 63
II – coronavirus-infected, n = 78
[1, 2, 3] - $P < 0.05$, comparison:
1 – healthy–healthy (different districts)
2 – healthy–infected (in the same district)
3 – infected–infected (different districts)

Table 3. **Distribution of immune biomarkers in healthy and coronavirus-infected bovine from different ecological districts**

Biomarker	District			
	Ukmergë		Trakai	
	I (n = 65)	II (n = 25)	I (n = 54)	II (n = 27)
Leukocytes (10^9/l)	6.2 ± 0.4	6.4 ± 0.3	4.9 ± 0.9[1]	6.3 ± 0.4[2]
Lymphocytes				
(%)	58.9 ± 2.9	57.2 ± 2.8	56.7 ± 2.1	55.9 ± 2.4
T	45.8 ± 0.7	44.3 ± 2.2	44.0 ± 2.3	42.7 ± 1.9
CD4	21.2 ± 1.6	21.1 ± 1.9	24.9 ± 2.1	18.9 ± 2.0
CD8	10.5 ± 0.7	10.9 ± 1.3	12.5 ± 1.5	13.4 ± 2.8
CD4/ CD8	2.2	1.9	2.0	1.3[1.3]

I – healthy,
II – coronavirus-infected
[1, 2, 3] – $P < 0.05$, comparison:
1 – healthy–healthy (different districts)
2 – healthy–infected (in the same district)
3 – infected–infected (different districts)

minants may lead to the induction of optimum immune responses to coronavirus (Anton et al., 1996).

In conclusion, our results show that the contaminated environment initiates the disturbance of the cellular immune system in the organism and favours transmission of bovine coronavirus infection.

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KORONAVIRUSAIS INFEKUOTØ GALVIJØ LÀSTELINIO IMUNITETO POKYÈIAI EKOLOGIÐKAI SKIRTINGUOSE LIETUVOS RAJONUOSE

S a n t r a u k a

Buvo tirti sveikø ir koronavirusais infekuotø galvijø hematologiniai ir làsteliniai imuniniai bioþymenys ekologiðkai skirtinguose Lietuvos rajonuose. Atlikus serologiná patikrinimà nustatyta, kad koronavirusais infekuoti galvijai sudarë 12,1% sàlyginai ekologiðkai ðvariame Ukmergës rajone (< 1 t/km^2/metus terðalø) ir 15,9% sàlygiðkai ekologiðkai uþterðtame Trakø rajone (60–62 t/km^2/metus terðalø). Sveikø ir koronavirusais infekuotø galvijø kraujyje sàlygiðkai ekologiðkai uþterðtame rajone eritrocitø, hemoglobino, leukocitø ir segmentuotø neutrofilø kiekiai buvo sumaþëjæ, tuo tarpu eozinofilø ir methemoglobino – padidëjæ. Làstelinio imuniteto tyrimai parodë, jog imunoreguliacinis indeksas (CD4 ir CD8 làsteliø santykis) Trakø rajono koronavirusais infekuotø galvijø organizme buvo sumaþëjæs (1,5 karto), palyginus su Ukmergës rajono koronavirusais uþkrëstø galvijø. Gauti rezultatai rodo, jog nepalankûs ekologiniai veiksniai veikia imunokompetentiniø làsteliø populiacijø persiskirstymà, imunodeficitinës bûsenos formavimàsi organizme ir virusiniø infekcijø paplitimà.