Prevalence of Viral Infections in Ecologically Different Districts and a New Method of Electrochemical Immunoassay

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The purpose of our research was to validate the impact of anthropogenic factors on the exposure to different viral infections, on the amount of circulating immune complexes in animals in ecologically different districts and create a novel, fast and cheap analytical method for determination of virus-induced bovine leukemia. Complex investigations of the immunocompetence in bovine organism demonstrated that the state of immune system is affected by harmful anthropogenic factors able to cause predisposition to mixed viral infections (bovine leukemia virus, coronavirus, rotavirus). In a conventionally ecologically contaminated district a markedly higher concentration of circulating immune complexes has been found in bovine blood serum. In this work, a novel amperometrical immunosensor for determination of virus-induced bovine leukemia was elaborated. The biological recognition part of this immunosensor is based on bovine leukemia virus antigens *gp51* cross-linked with glutaraldehyde on the tip of a graphite electrode. The detection method is based on a comparison of the amperometrical signals during a dynamic change of the potential before and after incubation of the immunosensor in the blood serum of neat investigated. The immunosensor can be successfully used for the diagnosis of bovine leukemia.

Key words: anthropogenic factors, circulating immune complexes, bovine leukemia virus, immunosensor

INTRODUCTION

The immune response is known to promote the protection of immunologically competent individuals from a majority of infections and environmental factors. The immune system acts as a self-recreating one (homeostatic), and it may come back to a normal level after a marked stimulation and regulation period. This self-regulation allows the sustainability of the organism despite different damaging factors of our environment having immunotoxic potential, harmful environmental factors among them (1, 2).

Over the recent years the immune system suppression of the mammalian organisms and immunodeficiency development have been discussed along with unfavourable factors of the ecosystem. Genetic peculiarities in the etiology and pathogenesis of certain diseases prove to be less significant than the surrounding of the organism (3–5). The majority of harmful chemical and physical factors possess a very broad spectrum of biological effects depending on their concentration and exposition duration.

One of the ways used for estimating the effect of harmful exogenic factors is investigation of the immune system of animals in ecologically different districts. Studies on the concentration of circulating immune complexes (CIC) are of great importance for monitoring the influence of anthropogenic factors, pathogenesis and treatment of different diseases. CIC have been described in a variety of clinical disorders such as rheumatological and autoimmune diseases, allergic diseases, viral, bacterial, and parasitic infections (6–8). In the pathogenesis of various infections, autoimmune, allergic and lymphoproliferative diseases, CIC are responsible for elimination of exogenous and endogenous antigens from the or-

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ganism. Immunoglobulin superproduction as the result of immune system disturbance is also eliminated by CIC.

The next important factor to characterise the influence of contaminants is prevalence of viral infections. Bovine leukemia virus (BLV) is an exogenous retrovirus known as the etiologic agent of enzootic bovine leukemia. BLV infects mainly B lymphocytes and establishes a persistent infection by integration of pro-viral DNA into the host genome (9). A manifestation of BLV-induced disease is an abnormal, persistent expansion of the peripheral B lymphocyte population, accompanied by high titres of antiviral antibodies directed against the main proteins of BLV (10). During BLV infection the immune response is characterised by both quantitative and qualitative changes in specific antibodies – IgG1, IgG2 and IgM isotypes (11). Detection of other viral infections such as corona-, rotaviruses is also important for monitoring the influence of anthropogenic factors. Therefore a fast, cheap and accurate diagnosis of viral diseases is very important.

Subsequently, BLV infection can be analysed using commercially available kits. Commercial BLV diagnostic kits usually consist of BLV antigens, control positive and negative sera. Antigens can be obtained from a BLV-infected and transformed cell culture. Control serum for this reaction can be prepared from serologically positive and negative bovine serum. Antibody response to BLV surface glycoprotein *gp51* may be detected soon after infection, but despite the presence of antibodies the character of disease is progressive (12).

Precipitating antibodies against the viral envelope proteins are easily scored using a relatively inexpensive immunodiffusion assay, however, enzyme-linked immunosorbent assays for envelope- or capsid-specific antibodies are more sensitive. Since animals may be seronegative in the earliest stages of infection, the most sensitive diagnostic technique is polymerase chain reaction (PCR). This reaction is used to examine seropositive and seronegative cattle for the presence of BLV DNA in bovine peripheral blood mononuclear cells and lymphocytes isolated from bovine spleen, bone marrow and the lymph nodes (12). The problem of the previously described method is that analysis takes a long time (from 4 h in the case of PCR to 48 h in the case of immunodiffusion reaction).

For acceleration of immunoassay, electrochemical immunosensors can be applied. Especially attractive are direct immunoassay-based immunosenors, because in use they do not need any additional immunochemicals and are based on a direct formation of antigen–antibody complex (13). Not long ago we have investigated a direct electrochemical immunosensor for detection of BLV (14). However, this immunosensor was not selective enough for BLV, because protein p24 which along with protein gp51 was used in previous immunosensor design is similar to proteins p24 which are persisting in other retroviruses like in human T cell leukemia virus type I (HTLV-1). It has been reported that already in denaturated state p24 shows cross-reactivity with anti-HTLV-1 goat serum (15). It was the reason for a decreased selectivity of the immunosensor based on a mixture of antigens (gp51 and p24) (14) to BLV, because the probability that antibodies against HTLV-1 can form complexes with p24 and influence the analytical signal was relatively high.

The purpose of our research was to validate the impact of anthropogenic factors on the exposure to different viral infections (bovine leukemia, corona- and rotaviruses) on the amount of circulating immune complexes in animals in ecologically different districts, and to elaborate as well as apply a new analytical immunosensor intended for determination of virus-induced bovine leukemia.

MATERIALS AND METHODS

Reagents

Antigens were obtained from BLV-producing cell culture fetal lamb kidney (FLK) and its clone FLK 44/2. Carbon rod ultra “F” electrodes (cat. No. 001281-10) 3 mm in diameter were purchased from SGL Carbon (Ringsdorff-Werke GmbH, Ringsdorff, Germany). Glutaraldehyde (25%) was purchased from Reanal (Budapest, Hungary). KCl, Na-acetate, KH_2PO_4, KOH, NaOH, $Na_2B_4O_7$, H_3BO_3 (analytical grade), polyethylenglycol 6000 (Serva, Heidelberg, Germany) and bovine serum albumin were obtained from Reachim (Kiev, Ukraine). All solutions were prepared by using HPLC grade water purified in a Purator-B Glass Keramic (Berlin, Germany), if not otherwise specified.

Equipment

All amperometric measurements were performed with a PA-2 polarograph (Laboratorny pristroje, Prague, Czech Republic) together with a three-electrode cell configuration consisting of a working carbon electrode, a saturated Ag/AgCl reference electrode and a Pt auxiliary electrode. The amount of CIC was determined on a CF-26 LOMO spectrophotometer (St. Petersburg, Russia).

Detection of viral infections

To detect the spread of viral infections, we have investigated 540 animals in ecologically different

districts. To detect BLV, we have used immunodiagnostic kits of the Institute of Immunology or those obtained from the Kursk Bioplant (Kursk, Russia) and Bovi Leuko Test from Institute Armand Frappier (Laval, Quebec, Canada). Corona- and rotavirus infections have been studied by the use of ELISA kits from Bio-X Diagnostics (Brussels, Belgium) and obtained at J. Kovalenko Experimental Veterinary Institute (Russia).

Preparation and determination of CIC by polyethylenglycol

We have studied CIC in healthy bovine organisms in conventionally clear (Ukmergė) and in ecologically contaminated (Trakai) districts. A total of 40 samples of sera were collected in Ukmergė and 32 samples in Trakai districts. CIC were isolated as described (16). For CIC separation from the serum proteins we used precipitation with 3% and 5% polyethylenglycol solution. The amount of CIC was determined spectrophotometrically (λ = 280 nm) and then expressed in mg/ml by using calibration curve.

Preparation of immunosensor

Graphite electrodes were polished and electrochemically cleaned as described (17). In this work, cross-linked antigen membrane was synthesised by cross-linking of *gp51* BLV antigens with glutaraldehyde vapor on the tip of graphite electrode according to the method for immobilisation described previously (18). Electrodes modified with antigens (modified electrodes) were stored in refrigerator at +4 °C.

Application of immunosensor in analysis

gp51 modified electrodes were inserted into 0.1 M phosphate buffer solution with 0.1 M KCl, connected into a three electrode circuit. The electrochemical equipment for electrochemical investigations mentioned in Equipment was used. The current was recorded during 100 mV/s potential scan from 0 to +600 mV *vs*. Ag/AgCl. After that the *gp*51-modified was incubated electrode for 30 min in the blood serum of the study cattle. Then, under the same conditions, the current during a 100 mV/s potential scan from 0 to +600 mV was registered and compared with the current obtained before incubation.

Determination of benz(a)pyrene and nitrates was carried out according to described methods (19, 20).

RESULTS AND DISCUSSION

Investigations of bovine CIC and the spread of viral infections have been carried out in the conventionally ecologically clearer Ukmergė district and the conventionally ecologically contaminated Trakai district.

While evaluating contamination of this country as a whole, in the conventionally ecologically contaminated Trakai district the total amount of contamination was found significantly higher than in the conventionally ecologically clear Ukmergė district (62 and 1 t/km^2/year, respectively) (20). In Trakai district the main wastes are nitrogen, sulphur, carbon and vanadium compounds belonging to class I–IV of harmfulness and having mutagenic and other negative effects on the organism. In Trakai district, a markedly higher concentration of benz(a)pyrene (table 1) and nitrates (table 2) has been detected.

In the conventionally ecologically clear district the CIC concentration in healthy bovine blood serum has been detected to range within the limits of 0.019–0.052 mg/ml, *i.e*. 0.034 ± 0.001 on the average; the coefficient of variability was 29%.

In the ecologically contaminated Trakai district a markedly higher concentration of benz(a)pyrene and nitrate, respectively, exerts an unfavourable effect on the immune system of the bovine organism. In this district the CIC concentration in healthy bovine blood serum ranged within 0.056–0.104 mg/ml, *i.e.*

Table 1. **Concentration of benz(a)pyrene in ecologically different districts**

Indices	Concentration of benz(a)pirene	
	Ukmergė	Trakai
	μg/kg	
Soil	2.67	67.2
Grass	1.02	3.2
Hay	3.35	7.6
Silage	7.71	80.0
Combined forage	0.20	10.8
	μg/l	
Water	<0.001	0.064
Milk	<0.001	0.014

Table 2. **Concentration of nitrate in ecologically different districts**

Indices	Concentration of benz(a)pirene	
	Ukmergė	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
Milk	10.5 ± 1.3	30.2 ± 2.6
Blood serum	37.5 ± 2.7	75.8 ± 5.6

0.072 ± 0.003 ($p < 0.05$); the coefficient of variability was 22%. In the conventionally contaminated Trakai district the concentration of CIC in bovine blood serum was twice as high.

Results of serological screening (N = 540) showed that in the conventionally ecologically contaminated Trakai district the spread of BLV-, corona-, and rotavirus-infected bovine was more significant than in the conventionally ecologically clearer Ukmergė district (Fig. 1). We have also detected that both in the conventionally unpolluted district and the contaminated one specific antibodies against corona- and rotaviruses are more frequently found in blood serum of BLV-infected animals (in Ukmergė district in healthy cows 0.96% and 3.12%, in BLV-infected cows 13.3% and 60%, respectively; in Trakai district in healthy cows 10.74% and 18.12%, in BLV-infected cows 7.35% and 69.12%, respectively).

The results of our investigation show that in the conventionally ecologically contaminated Trakai district the number of animals infected with BLV, corona- and rotaviruses is higher than in the conventionally ecologically clear district.

During this research we have used antigen gp51 to improve the selectivity of the above-mentioned immunosensor for diagnosing BLV (14). In this immunosensor we have used another electrochemical detection method – we registered currents during 100 mV/s potential sweeps from 0 to +600 mV *vs.* Ag/AgCl. It means that electrochemical detection was at least 4 times shorter. In the immunosensor described in (14), at least two complete cyclic voltammograms were registered for the same purpose. Investigation of the immunosensor was performed according to the protocol described in Experimental.

The same gp51-modified electrode after incubation in the blood serum of infected bovine, dipped in the same 0.1 M phosphate buffer solution shows a bigger evolution of currents during potential scan from 0 to +600 mV *vs*. Ag/AgCl (Fig. 2, whole lines) than before incubation (Fig. 2, dotted lines).

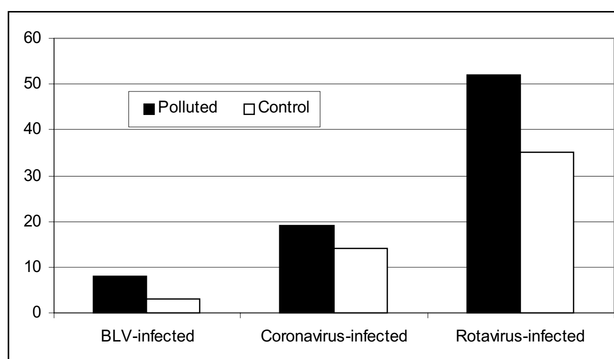

Fig. 1. Prevalence of viral infections in animals

If BLV-infected blood serum was investigated, the increase in currents was higher than 20% in comparison with currents obtained before incubation. If the antigen-coated electrode was incubated in the blood serum of healthy bovine, differences between the currents registered before and after incubation were less than 2% (data not shown). This phenomenon shows that differences in registered currents arise because the capacity/impedance of the immunosensor has changed after interaction of antibodies against glycoprotein gp51 present in the blood serum of BLV-infected neat with antigens immobilised on the graphite electrode.

It means that comparison of currents obtained during potential scan from 0 to +600 mV can be used as a registration method for detection of antigen–antibody interaction. The best results were obtained at a scan rate of 100 mV/s (Fig. 2). There are a few reasons to use a faster potential scan rate: (i) differences between the currents obtained before and after incubation are more significant than in the cases of lower sweep rates; (ii) the experiment is faster.

The differences between the currents obtained were significant and were used as an indication of bovine leukemia. The results were compared with those obtained by a standard BLV detection method (immunodiffusion reaction) with a BLV diagnostic kit. During investigation of 10 BLV-infected and 25 BLV-noninfected blood sera, no detection mistakes were obtained with the immunosensor described. On the other hand, it was found that after investigation of blood serum of BLV-noninfected cattle the electrodes can be used for further detections.

However, detection of BLV directly in the blood or blood serum of infected cattle was impossible, because the electrochemical signals were significantly influenced by a high concentration of electro-active compounds such as ascorbic and uric acids. Regeneration of this immunosensor by using high (1M) ionic

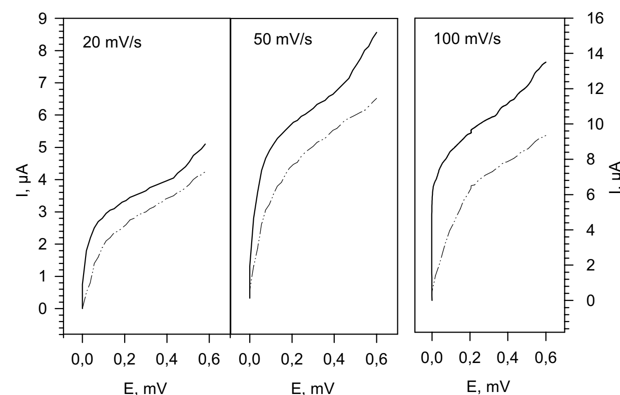

Fig. 2. Measurements of current during potential scans applied to immunosensor: *1* – before, *2* – after incubation in blood of BLV infected cattle

strength, low or high pH (1–4 and 9–13) and 0.5 M glycine solutions was also impossible because of a very strong complex between gp51 and anti-gp51 antibody. It means that gp51 graphite electrodes can be used only until a first positive detection of BLV infection.

However, we think that both above-mentioned disadvantages are not so significant: (i) because, as it was shown during investigations, the described registration of currents can be performed in the same buffer solution under the same conditions before and after incubation in the blood serum of cattle; (ii) graphite electrodes are cheap and can be easily coated with a new gp51 layer.

We think that the electrochemical method described above can be used also for diagnosing other virus-induced diseases.

CONCLUSIONS

In the conventionally ecologically contaminated Trakai district a twice higher concentration of CIC has been found in bovine blood serum and more animals infected with BLV, corona- and rotaviruses have been detected as compared to a conventionally ecologically clear district.

During this research we have improved the immunosensor applied for detection of virus-induced diseases in two aspects: detection time wis shorter, the selectivity for BLV has improved.

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VIRUSINIŲ INFEKCIJŲ PAPLITIMAS EKOLOGIŠKAI SKIRTINGUOSE RAJONUOSE IR NAUJAS ELEKTROCHEMINIS IMUNOTYRIMŲ METODAS

S a n t r a u k a

Šio darbo tikslas – įvertinti antropogeninių veiksnių įtaką virusinių infekcijų paplitimui, cirkuliuojančių imuninių kompleksų kiekiui galvijų organizme skirtingos taršos rajonuose ir sukurti naują, greitą ir pigų analitinį metodą viruso sukeliamai leukemijai nustatyti.

Kompleksiniai galvijų imunokompetencijos tyrimai rodo, kad antropogeniniai veiksniai daro įtaką mišrių virusinių infekcijų (galvijų leukemijos, corona, rotavirusų) paplitimui bei gerokai padidina cirkuliuojančių imuninių kompleksų kiekį.

Sukurtas naujas amperometrinis imunosensorius galvijų leukemijai nustatyti. Leukemijos viruso antigenas *gp 51* glutaro aldehido garais imobilizuojamas anglinio elektrodo paviršiuje. Takėme amperometrinius signalo registravimo metodus, lyginome signalus, gautus prieš inkubaciją ir po jos infekuoto galvijo kraujo serume.

Raktažodžiai: antropogeniniai veiksniai, cirkuliuojantys imuniniai kompleksai, galvijų leukemijos virusai, amperometrinis imunosensorius