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LEUKOGENIC PROPERTIES OF PURIFIED BLV AND POSSIBLE ROUTES OF ITS TRANSMISSION WITH BLOOD CELLS PRESENT IN MILK

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Résumé

PROPRIETES LEUCOGENES DU BLV PURIFIE ET VOIES POSSIBLES DE TRANSMISSION PAR LES CELLULES SANGUINES PRESENTES DANS LE LAIT. — Les résultats d'une étude de cinq ans sur la possibilité de l'induction expérimentale de la Leucose Bovine avec du virus purifié et différentes substances contenant du BLV ont montré que l'inoculation de veaux avec du BLV purifié (particules d'une densité de 1,14 à 1,17 dans un gradient de sucrose 20-60 %) peut provoquer l'infection par l'oncornavirus et la Leucose Bovine. Parmi 41 veaux infectés expérimentalement, 9 ont fait de la Leucose. Cinq de ces 9 veaux provenaient d'un groupe de 22 inoculé avec du virus purifié. Le diagnostic de leucose chez ces 9 veaux fut porté par hématologie (3 animaux) et par histologie (6). Le BLV a été isolé des leucocytes de tous ces animaux.

On a également montré que le développement de l'oncornavirus précède le développement de la Leucose Bovine, mais seulement une petite partie des animaux infectés par l'oncornavirus et présentant des anticorps ont développé des formes cliniques typiques d'hémoblastose.

On a aussi recherché le rôle du lait et des cellules sanguines qu'il contient dans la Transmission de la Leucose Bovine. Il y avait dans le lait des structures de type oncornavirus antigéniquement identiques au BLV, susceptibles de se reproduire dans les cellules sanguines présentes dans le lait.

Ces résultats, ainsi que la transmission expérimentale de la Leucose Bovine chez des veaux en les élevant avec du lait cru de vache leucosique, permet d'en déduire des voies possibles de contamination naturelle.

Introduction

Successful reproduction of bovine leukemia transmission to cattle and sheep with different materials from cattle with leukemia (blood, lymphocytes, suspension of lymph node cells,

leukocyte cultures, etc.) does not yet prove the role of BLV (bovine leukemia virus) in the etiology of bovine leukemia. Only reproduction of experimental leukemia with purified BLV or ultrafiltrates of different BLV-containing materials can demonstrate BLV-etiology of this disease.

Table 1. — Results of cattle investigation

Transmission materials	Number of animals	Clinical signs	Hematology	Leukocyte cultures		
				Structures ^a	Reverse-transcriptase activity ^b	Antigen identity with BLV (IDT)
Purified BLV isolated from cattle with leukemia	22	3-/22	2+/22 3±6 3-/19	13+/	2+/10	7+/10
Purified BLV inactivated by heating	3	1-/3	3-/3	1+/ 2-/3	Not tested	1+/1
BLV containing Featal Lamb spleen culture medium	12	2-/12	4+/12	5+/7	Not tested	³ +/ 1-/7
Blood from cattle with leukemia .	2	2-/2	2-/2	2+/2	2-/2	2+/2
Mycoplasma	2	2-/2	2-/2	2-/2	Not tested	2-/2
Total	41	6-/41	6+/41	21+/ 5-/33	12+/ 2-/4	8+/9 1-/11
Control animals	= 9	9-/9	9-/9	2-/9	Not tested	Not tested

^a: of 1.14-1.18 g/ml density ; ^b: of 1.14-1.18 g/ml structures

In this connection the present investigation was undertaken to study the possibility of reproduction oncornavirus (BLV) infection and leukemia in newborn cattle with purified BLV and investigate the comparative development of oncornavirus infection and leukemia in cattle infected with purified BLV or different BLV-containing materials. The present investigation was also undertaken to search for BLV in leukemic cattle milk, to study the possibility of BLV-reproduction in cells penetrating into milk from blood, and to demonstrate the possible role of leucocytes presenting in milk in the transmission BLV from leukemic cattle to healthy animals.

Materials and methods

BLV and BLV-containing materials used for experimental reproduction of bovine leukemia

a) BLV was isolated from leukocyte cultures and primary trypsinized cell cultures of lymph nodes and spleen from cattle with acute and chronic leukemia (Parfanovich *et al.*, 1973 ; Zhdanov *et al.*, 1975, Parfanovich *et al.*, 1977). For experimental inoculation the structures of 1.14-1.18 g/ml density in 20/60 % sucrose gradient, isolated from culture media, concentrated 2.000-5.000 times with differential and gradient centrifugation were used. The

in the reproduction of bovine leukemia experiment

Cultures of cells from lymph nodes and spleen			Serology				
			Antigen BLV		Antibody to BLV		
Structures ^a	Reverse transcriptase activity ^b	Antigen identity with BLV (IDT)	IDT	IDT	CFT	Necropsic diagnosis	Histological diagnosis
6+/6	3+/	2+/2	19+/22	12+/19	14+/19	5+/12	4+/12
Not tested	Not tested	Not tested	1+/3	3-/3	3-/3	Not tested	Not tested
Not tested	Not tested	Not tested	3+/12	9+/12	10/12	Not tested	Not tested
2+/2	Not tested	2+/2	2+/2	2+/2	2+/2	2+/2	2+/2
1-/1	Not tested	1-/1	1-/1 1-/2	2-/2	2-/2	1-/1	1-/1
3+/	4+/5 3-/6	25+/	23+/38 1-/41	26+/38	7+/15	6+/15	
Not tested	Not tested	Not tested	3+/9	9-/9	9-/9	Not tested	Not tested

amount of BLV injected into experimental animals was established by spectrophoto-assay. 2 ml of injected BLV (optimal dose for baby calves) contained 35 mg of protein.

a) Purified BLV, inactivated with heating (60 °C, 30 min).

b) Ultrafiltrate of BLV-containing FLS (fetal lamb spleen) culture medium. FLS-culture was presented by Dr Van Der Maaten (National Animal Disease Center, Ames, Iowa, USA).

d) The whole blood from a cow with leukemia (179.000 leukocytes ml, 96 % lymphocytosis) was inoculated into 2 calves (50 ml intramuscularly).

e) *Mycoplasma* isolated from the same cultures as BLV.

f) BLV-containing milk from cattle with leukemia was administrated by feeding.

Animals

a) For the bovine leukemia transmission experiment 50 baby calves (1-40 days) of Estonian red, Estonian black-particoloured and Estonian aboriginal breed were used. All of them were received from herds and farms without leukemia. Before infection the clinical, hematological, immunological and virological investigation of newborn calves and their mothers were carried out.

Table 2. — Investigation of leukemic cattle — milk donors.

Animals	Hematology			Leukocyte cultures			Leukocytes of blood		Serology IDT	
	Leukocyte number thousand/ml	% of lymphocytes	Structures ^a	Reverse-transcriptase activity ^b	60-70S RNA from 1.14-1.18 g/ml structures	Antigen identity ^c	reverse transcriptase activity associated with 60-70S RNA	Antigen BLV	Antibody to BLV	
1042	26.000	88	+	Mg ²²	-	+	+	+	+	
1043	21.500	87,5	+	Mg ⁺⁺	+	+	+	+	+	
« Teresino »	28.000	89	+	Mg ⁺⁺	+	+	+	+	+	
« Tirva 1 »	60.000	91	+	Mg ⁺⁺	Not tested	+	Not tested	-	+	
« Tirva 2 »	123.000	93	+	Mg ⁺⁺	+	+	+	+	+	
« Uchkhoz »	N	N	-	-	-	-	-	-	-	
« Estonia »	N	N	-	-	-	-	-	-	-	

^a: of 1.14-1.18 g/ml density; ^b: of 1.14-1.18 g/ml structures; ^c: of 1.14-1.18 g/ml structures with BLV.

41 calves were infected. 9 calves were retained as control animals.

b) The cattle with typical leukemia were used for isolation of BLV (Parfanovich *et al.*, 1974; Zhdanovet *et al.*, 1974) and as donors of milk (Parfanovich *et al.*, 1978). The characteristics of leukemic cattle-donors of milk is described in Table 2.

Organization of experiment on bovine leukemia reproduction

The experimental inoculation of baby cattle with purified BLV and other BLV-containing materials were carried out on the base of the Estonian Agricultural Academy (Tartu) in 1973-1974. The purified BLV was inoculated into 22 baby calves intravenously, intraster-nally, in the area of preshoulder lymph-nodes, subcutaneously, etc.; 3 calves received purified BLV inactivated; 13 baby cattle received the ultrafiltrate of BLV-containing FLS-culture medium (150-250 ml) intraperitoneally; the whole blood from leukemic cattle was injected 2 calves (50 ml) intramuscularly. Micoplasma was inoculated in 2 animals into lymph nodes.

The transmission experiment with milk from leukemic cattle was based in the Belotserkovsky Agricultural Institute (Belaya Tserkov) in 1970. 3 groups of baby-calves were used: The 1st group (animals N° 1 and 2) were born from leukemic cows and fed with milk of the same cows.

The 2nd group (N° 3 and N° 4 animals) were born from healthy cows but fed with milk of leukemic cattle.

The 3th group (N° 5 and N° 6 animals) were born from leukemic cattle and fed with milk of healthy cow.

All experimentally inoculated animals were studied with regularity (3-4 months) by clinical, hematological, virological, serological and molecular-biological methods, and in the case of enforced or diagnostic slaughter by pathological and histological test.

The methods of investigation of cattle with leukemia, experimentally inoculated and healthy control animals

Cell lines

a) Short term cultures of leukocytes. Blood was collected from jugular vein over EDTA or heparine. Leukocytes were isolated by technique of Odajima and Sonoda (1971). the

amount of cells was about 3.0×10^6 /ml of Eagle MEM with 20 % fetal calf serum.

b) Primary trypsinized cell cultures of lymph nodes, spleen and kidney were prepared in the case of enforced or diagnostic slaughter. They were propagated in Eagle MEM-199 medium with 0.5 per cent hydrolysate of lactalbumin equal volumes and 10-20 per cent of calf serum.

c) Short term cultures from cells, isolated from milk. Milk was diluted with 0.83 ammonium chloride in equal volume and this mixture was centrifuged 2.000 rpm for 10 min. the pellet was resuspended in 30 % Eagle MEM, 30 % 199 medium and 30 % of 0.5 % hydrolysate of lactalbumin with 10 % of calf serum and washed off twice in the same medium by the same centrifugation procedure. The amount of cells were $3-4 \times 10^6$ in ml.

All cell cultures contain 1.000 units of penicillin and 1.000 mg of streptomycin per ml of medium.

Labelling, isolation and purification of BLV from cell cultures medium in sucrose gradient

For the study of RNA-containing viruses the cell-cultures were labeled with ^3H -uridine (10-20 mCi/ml, specific activity 26 Ci/mmol) for 24 hours. The culture medium was collected, cell debris was removed by centrifugation at $15,000 \times g$ for 20 minutes, and the virus was sedimented at $100,000 \times g$ for two hours. the pellet was collected, resuspended in THE-buffer (Tris-HCl 0.01M pH 7.4 ; NaCl 0.1m, EDTA 0.001M) layered onto a linear sucrose density gradient 20-60 per cent (w/w) and centrifuged in a SW 27.1 rotor of a Spinco L-575 centrifuge at 25,000 rpm for 3 hours. The gradient fractions were collected in a LKB fractionator and the radioactivity of the acid insoluble material was measured in a Packard-Tricarb liquid scintillation counter. Unlabeled virus for electron microscopy, reverse transcriptase reaction and for experimental inoculation of animals was also concentrated and purified as described above.

Isolation of BLV from milk

Isolation of structures of 1.14-1.18 g/ml density from milk carried out by the method of Moore (1972) in modification of Schlom and Spiegelman (1971) and by Howk (1973).

Isolation and analysis of BLV RNA

^3H -uridine-labeled virus was purified in 20-60 per cent sucrose density gradients. The fractions with density of 1.14-1.18 g/ml were

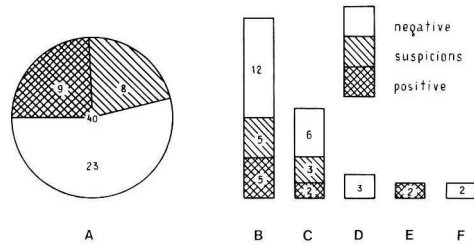


Fig. 1. — Results of bovine leukemia transmission experiment on cattle.

A. Results of investigation of all experimentally inoculated animals.

B. Animals, experimentally inoculated with purified BLV.

C. Animals, inoculated with BLV-containing FLS culture medium (celle free).

D. Animals, inoculated with inactivated by heating purified BLV.

E. Animals, infected with the whole blood of leukemic cattle.

F. Animals, infected with purified micoplasma.

The digits show the number of animals.

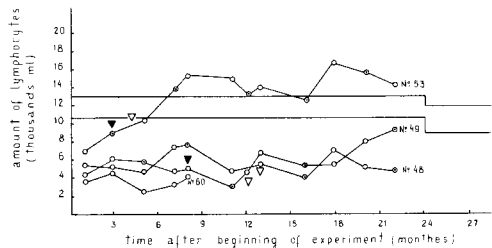


Fig. 2. — Results of haematological, virological and immunological investigation of cattle No. 48, 49, 53, 60, experimentally inoculated with BLV-containing FLS culture medium.

Abscissa — lymphocytosis (thousands/ml).

Ordinate — time after beginning of experiment (months).

(○) production of BLV by short term leukocytes cultures

(▲) beginning of the appearance of BLV-antigen in serum

(△) beginning of the appearance of antibody to BLV in serum.

collected, diluted with the buffer to a density of 1.10 g/ml and the material was pelleted at $15,000 \times g$ for 1 hour. RNA was extracted with phenol and sodium dodecyl sulfate and studied in sucrose density gradient 10-30 per cent (w/v).

Reverse transcriptase assay

To study the enzymatic activity of the extracellular virus the latter was isolated from non-labeled cultures as described above. The material from sucrose gradient fractions with a density of 1.14-1.18 g/ml was pelleted and used in the reverse transcriptase reaction. The reaction was performed with endogenous template and also with an exogenous template oligo (dT).poly (rA).

The incubation mixture for the reaction with endogenous template contained in 1ml : 10 μ mole pH 8.3 Tris-HCl, 150 μ mole NaCl, 5 μ mole $MgCl_2$ (or 0.125 μ mole $MnCl_2$), 5 μ mole dethiothreitol, 0.015 per cent non-ionic detergent NP-40, 0.1 μ mole of each deoxynucleoside triphosphate, dATP, dGTP, dCTP, 10 μ Ci³H-TTP (specific activity 16 Ci/mmole), and virus 35-50 μ g of protein. The reaction was

stopped by the addition of 10 volumes of 10 per cent TCA in 0.1M pyrophosphate.

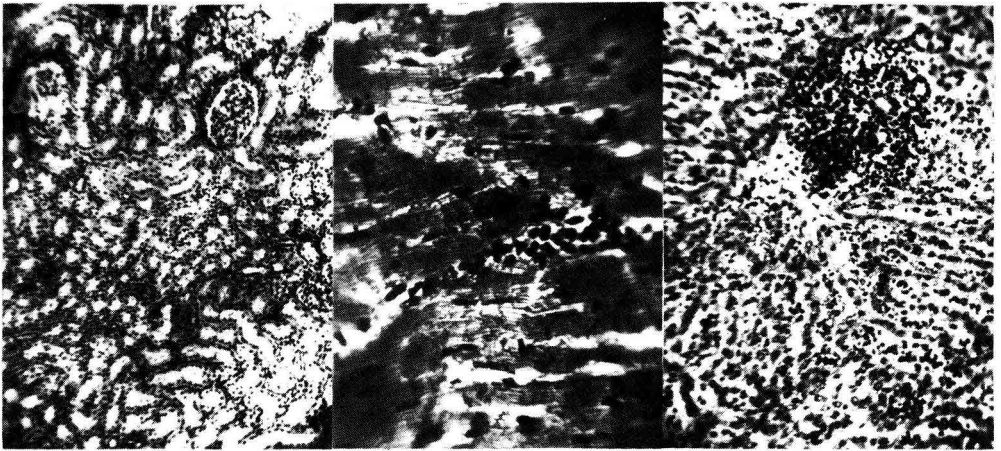
The incubation mixture for the reaction with exogenous template contained in 1 ml : 50 μ mole Tris-HCl, pH 7.8, 60 μ mole KCl, 5 μ mole $MgCl_2$ (or 0.125 μ mole $MnCl_2$), 2 μ mole dithiothreitol, 0.05 per cent Triton X 100, oligo (dT) : poly (rA) 0.02 A_{760} , 1 μ Ci³H TTP, virus 35-50 μ g of protein.

We also have studied the intracellular viral reverse-transcriptase activity (synthesis of ³H-TTP product) associated with 70-60S RNA as described by Schlom and Spiegelman.

Study of morphology and viability of cells presenting in milk

Thick smears were prepared from cells presenting in milk as described by Kondrakhina (1977, 1978). They were fixed by methanol (5 min) and stained by Romanovsky-Gimsa. For study the viability of cells the tripan-blue staining (0.4 % pH 7.5) was used.

For the same purpose the precursor of DNA synthesis ³H-thymidine was added to short-term milk cell cultures.



A

B

C

Fig. 3. — Lympholeukosis.

A. Kidney of three years cattle No. 1, inoculated with purified BLV at the age of 10 days. Diffuse and local lymphocells infiltration ($\times 130$).

B. Heart muscle of 3 years cattle No. 11 inoculated with purified BLV at the age of 10 days. Infiltration of lymphoid cells ($\times 260$).

C. Liver of two years cattle No. 18 inoculated with the whole blood of leukemic cattle at the age of 12 days ($\times 260$). Staining with hemato xylin-eosin.

The immunodiffusion test (IDT)

The IDT was carried out for detection of BLV-antigen and antibody to BLV Plates contained 0,8-1,0 % agarose gel in 0,05M Tris HCl pH 7,2 with 0,85 % NaCl (Miller *et al.*, 1974 ; Ressang *et al.*, 1974, 1975 ; Miller and Van Der Maaten, 1976). The antigen consisted of purified BLV (structures of 1.14-1.18 g/ml in 20-60 % sucrose gradient) isolated from BLV-containing culture medium FLS, FLK (fetal lamb kidney) and FBT (fetal bovine thymus) and treated with Tween-80 and ether.

Rabbit anti-purified BLV serum was prepared by immunization of rabbit into popliteal lymph-node with Freund's adjuvant. This anti-BLV rabbit serum was absorbed with fetal calve serum and its specificity was demonstrated in IDT with reference antigen from the National Animal Disease Center, Ames, Iowa

(Parfanovich *et al.*, 1976 ; Zhdanov *et al.*, 1976).

Micro-complement fixation test

This test was applied as described earlier (Miller *et al.*, 1974, in the modification of Ressang *et al.*, 1976). The antigen also consisted of purified BLV structures with 1.14-1.18 g/ml density, isolated from FLS, FLK and FBT and treated with Triton X-100.

Results

The experimental reproduction of oncornavirus (BLV) infection and bovine leukemia in calves with purified BLV and others BLV-containing materials (Table 1 and Figs. 1, 2, 3, 4, 5, 6)

The clinical investigation of experimentally

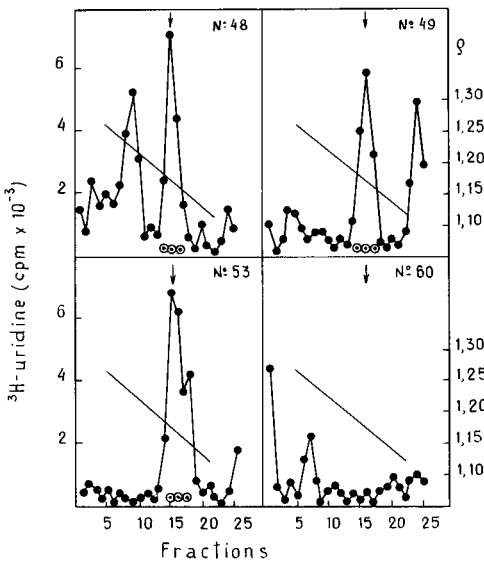


Fig. 4. — Density distribution in 20-60 % sucrose gradient of ^3H -uridine labelled structures from short term leukocyte cultures medium of calf No. 16, experimentally inoculated with purified BLV (A) and control calf No. 14 (B) and also primary cell cultures of lymph node of the same animals — calf No. 16 (C) and No. 14 (D).

(○) antigenical identity with BLV.

(●) reverse transcriptase activity.

The conditions of centrifugation : 24.000 rpm, 4 hours, 4 °C, rotor SW 25,1 of Spinco L5-75 (« Beckman », USA).

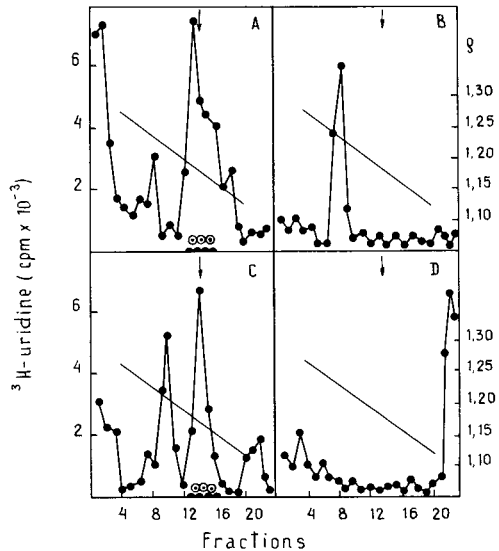


Fig. 5. — Density distribution in 20-60 % sucrose gradient of ^3H -uridine labelled structures from short term leukocytes cultures medium of calves No. 48, 49, 53 and 60, experimentally inoculated with BLV containing FLS cell culture medium (cell free).

(○) antigenical identity with BLV.

The conditions of centrifugation as in Fig. 3.

inoculated cattle demonstrated some retardation in common development in comparison with control animals. Some cattle had remarkable enlargement of lymph nodes: retropharyngeal, cervical, prescapular, popliteal, supraudal, etc. Some of them had double exophthalmos.

The haematological investigation showed subleukemic tendency to disease in the case of most experimentally inoculated animals. Only some cattle experimentally inoculated with BLV-containing FLS culture medium, had leukocytosis (16.000-19.000 in ml) and lymphocytosis (74 %-86 %) (Fig. 2), already present in 150-200 days after infection. The histological investigation of lymph nodes, spleen, marrow, heart, liver of 15 animals, killed three years

post infection, confirmed that 6 cattle had lympholeukosis. 4 of them were inoculated with purified BLV and 2 with the whole blood of leukemic cattle (Fig. 3). 2 of 15 animals were suspicious.

Both the short-term leukocyte cultures and primary trypsinized cell cultures of lymph nodes, spleens and kidneys of most of the experimentally inoculated animals produced structures of 1.14-1.18 g/ml density in 20-60 % sucrose gradient in the culture medium. The structures possessed reverse transcriptase activity and were antigenically identical to BLV (Fig. 4, 5, 6). These oncornavirus structures of 1.14-1.18 g/ml density have a high molecular weight RNA with a sedimentation constant of about 60-70S and also some slow sedimenting components.

Reverse transcriptase activity associated with 60-70S RNA of intracellular virus was tested in short term leukocyte cultures, primary trypsinized cultures of lymph nodes and spleen and also in leukocytes of blood.

By using the immunodiffusion test and rabbit anti-BLV serum it was demonstrated that

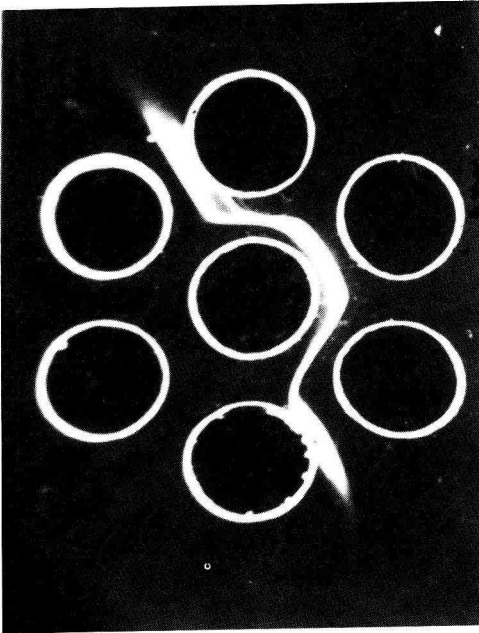


Fig. 6. — IDT demonstration of antigenic identity of BLV isolated from cattle, experimentally inoculated with purified BLV and BLV, isolated from spontaneous leukemic cattle.

The wells 1, 2, 3 — rabbit antiserum to disrupted BLV, absorbed with normal calf serum.

The wells 4, 6 — disrupted BLV structures of 1.14-1.18 g/ml density, isolated from experimentally inoculated calves No. 18 and No. 34.

The well 5 — normal calf serum.

The well 7 — disrupted BLV structures of 1.14-1.18 g/ml density isolated from spontaneous leukemic cattle.

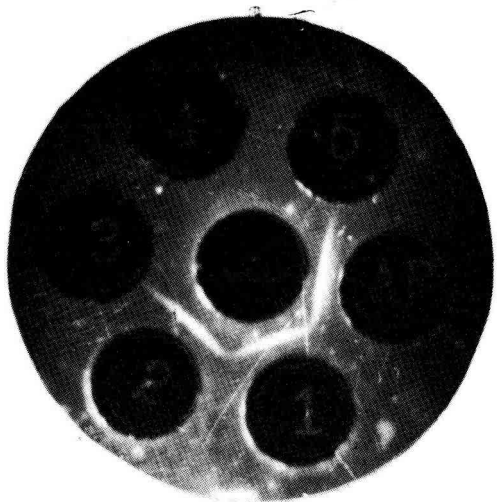


Fig. 7. — Study of antigenic identity of 1.14-1.18 g/ml density structures isolated from milk of leukemic cattle (hollow No. 1 and 2), from milk of healthy cattle (No. 3), milk from trade (No. 4) and boiled milk of leukemic cattle (No. 5).

The central well — rabbit anti-BLV serum absorbed with normal fetal calf serum.

the serum of 25 experimentally inoculated animals contained specific antigen, but only the sera of 5 animals with histologically confirmed diagnosis of lympholeukosis has this antigen constantly. The antibodies were not regularly found in serum of experimentally inoculated animals. 23 cattle had antibodies as it was shown by immunodiffusion test, and 26 cattle also had antibodies as demonstrated by complement fixation test.

2. BLV presence in leukemic cattle milk ; and its reproduction in blood cells presenting in milk.

The leukemic milk donor cattle had 20.000-123.000 leukocytes in ml of blood and lymphocytosis was about 86-92 %. The short term leukocytes cultures of these milk-donors produced, in the culture medium, structures of 1.14-1.18 g/ml density in sucrose gradient that possessed reverse-transcriptase activity and antigen identical with BLV. Reverse transcriptase activity associated with 60-70S RNA was tested in leukocytes. The serum of donor cattle contained BLV-antigen and antibodies to BLV (Table 2).

The investigation of leukemic cattle milk demonstrated the presence of BLV in it.

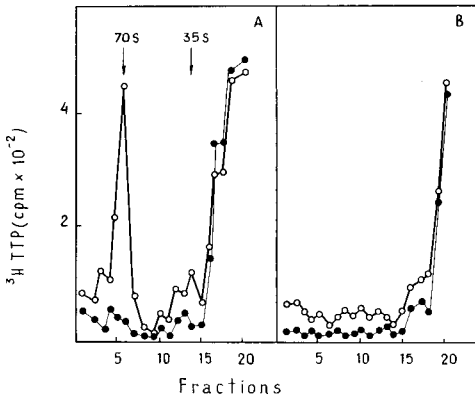


Fig. 8. — Sedimentation profiles of $^3\text{H-TTP}$ labeled product of reverse-transcriptase reaction with intracellular structures of milk cells from leukemic cattle — milk donors (A) and healthy control cattle (B).
 (○—○) without RNA-ase treatment
 (●—●) with RNA-ase treatment
 Condition of centrifugation : 38.000 rpm, 3 hours, + 28 °C, in rotor SW40 of Spinco L575 (Beckman, USA).

Structures of 1.14-1.18 g/ml density were antigenically identical to BLV by IDT (Fig. 7). The reverse transcritase activity of these structures was weakly positive. Index of the reaction was sometimes 2.7-3.0, but more often 2.0-2.1 (Table 3).

The study of healthy cattle raw milk and pasteurized milk from retail trade gave negative results.

The antibody to BLV was not found in leukemic nor in healthy cattle milk.

It was found that milk of both healthy and leukemic cattle contain different cells, such as : epithelial, mioepithelial, foamy cells, histiocytes, macrophages, neutrophils, eosinophiles, lymphocytes and also free nuclei.

The amount of blood cells in milk of leukemic cattle was about 36.000-40.000/ml i.e. 25 % of the total. The amount of the cells in milk varied and depends on the amount of leu-

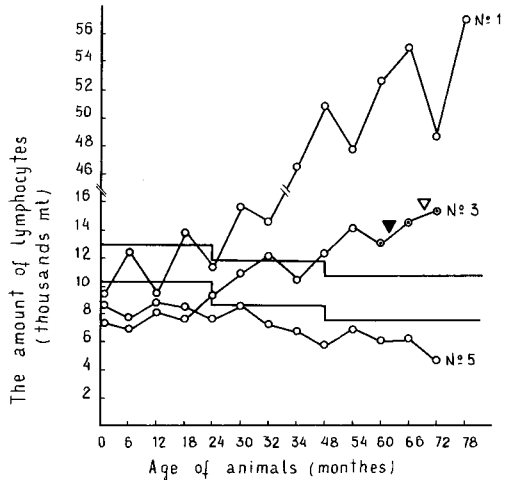


Fig. 9. — Results of haematological, virological and immunological investigation of cattle No. 1, No. 3, and No. 5 from the bovine leukemia transmission experiment by feeding colostrum and milk of leukemic cattle.
 Abscissa — lymphocytosis (thousands/ml).
 Ordinate — time after beginning of experiment (months).
 (○) production of BLV by short term leukocytes cultures
 (▼) discovery of BLV-antigen
 (∇) discovery of antibody to BLV
 The cattle No. 1 was not tested virologically serologically.

Table 4. — Investigation of role of milk in the experiment on bovine leukemia induction.

Animals	Haematology		Leukocyte cultures		Leukocytes of blood		Cultures of lymph. nodes and spleen	Serology IDT
	Leukocyte number thou- sands/ml	% lymphocytes	Structures of 1.14-1.18 g/ml	Reverse-transcrip- tase activity of 1.14-1.18 g/ml structures with BLV	Reverse-transcrip- tase activity associated with 60-70S RNA	Structures of 1.14-1.18 g/ml density	Reverse-transcrip- tase activity of 1.14-1.18 g/ml structures with BLV antigen	Antibody to BLV
Animals born by healthy cattle and fed with milk of cattle with leukemia :								
No. 3	15700 (Mg ⁺⁺)	89 +	+ +	+ /Mg ⁺⁺ /	+ +	+ +	+ +	-
No. 4	14900 (Mg ⁺⁺)	92 +	+ +	+ /Mg ⁺⁺ /	+ +	+ +	+ +	-
Animals born by cattle with leukemia, but fed with milk from healthy cattle :								
No. 5	9200	62	-	-	-	-	-	-
No. 6	8700	63	-	-	-	-	-	-

kocytes and per cents of lymphocytes in blood.

By vital staining with Tripan-Blue the viability of most of the cells presenting in milk was demonstrated. Labelling of the cells with DNA-synthesis precursor ^3H -thymidine confirmed this. Short boiling (1-2 sec.) of milk, as a rule leads to an increase of dead cells.

The short-term cultures of leukemic cattle milk cells produced ^3H -uridine labelled structures of 1.14-1.18 g/ml density. The identity of them with BLV was shown by IDT, the reverse transcriptase ability of these unlabelled structures was rather weak and Mn^{++} -dependant. The reverse transcriptase activity associated with 60-70S RNA in the homogenate of leukemic cattle milk cells was more definite (Fig. 8).

The cell cultures of healthy cattle milk did not produce oncornavirus structures.

3. *The experiment of bovine leukemia transmission by feeding baby-calves with colostrum and milk.*

The animals of the first group (N^o1 and N^o 2), which were born from leukemic cattle and fed with colostrum and milk of their mothers in 16-18 months after beginning of this experiment had typical bovine leukemia changes in blood. One of these animals died at two years old and the other died at seven years with leukocytosis (50.000-86.000 in ml) and lymphocytosis (80-90 %). Histological study of the materials from both of them confirmed the diagnosis of bovine leukemia. Unfortunately the virological and serological study was not carried out on the blood lymphocytes and serum of cattle N^o 1 and N^o 2.

The animals of the second group (N^o3 and N^o4), that were born from healthy cows, but fed with colostrum and milk from leukemic cows had lymphocytosis to 90 % at the age of two years. In the subsequent years these animals had typical bovine leukemia blood changes. The short term cultures of blood leukocytes of these animals produced BLV (the oncornavirus structures of 1.14-1.18 g/ml density, possessing rather slow reverse transcriptase and Mg^{++} -dependant activity and being antigenically identical to BLV). The serum of these cattle had BLV antigen and antibody (Table 4, Fig. 9). 6 years after beginning of these transmission experiment the animals N^o3 and N^o 4 were killed and investigated by virological and histological methods. The pri-

mary trypsinized cultures of lymph nodes and spleen also produced BLV. Histological changes confirm the diagnosis of lymphoid leukosis.

The animals of the third group (N^o5 and N^o6) that were born from leukemic cattle but fed with colostrum and milk of healthy animals had slight changes of leukocytosis and lymphocytosis during the first three years. In the following years the amount of leukocytes and per cent of lymphocytes became stable within the normal limit. The short-term leukocytes and primary trypsinized lymph nodes cultures did not produce BLV. The BLV antigen and antibody in the serum were not demonstrated (Table 4).

Discussion

The attempts to transmit experimental bovine leukemia by using different materials of leukemic cattle were made by many investigators and most of them obtained positive results (Rosenberger *et al.*, 1968 ; Miller *et al.*, 1972, Hoss and Olson, 1974, Olson *et al.*, 1972, 1973 ; Wittman and Urbanek, 1969 ; Ressang, 1976 ; Filatov *et al.*, 1976). But for establishment of BLV's role in etiology of bovine leukemia it is necessary to obtain positive transmission experiments using purified BLV.

Our results of a five years bovine leukemia transmission experiment demonstrate that inocuation of purified BLV (the structures of 1.14-1.18 g/ml in 20-60 % sucrose gradient), isolated from leukemic cattle, and also other BLV-containing materials (BLV-containing FLS culture medium free of cells, blood of leukemic cattle) lead to bovine leukemia reproduction in some animals. In our experiment there were 9 cattle with bovine leukemia of 41 experimentally inoculated. The diagnosis of bovine leukemia was confirmed by histological investigation in six cases. Four animals of these six cases were infected with purified BLV and two with whole blood. The diagnosis was established in three cases of nine animals hematologically. BLV was reisolated not only from these nine animals, but also from most of the experimentally inoculated animals.

But our results show comparatively low oncogenicity of purified BLV. Only five animals became ill out of 22 experimentally inoculated with purified BLV. Perhaps, this is connected

with conditions of BLV purification as the reverse-transcriptase activity of purified BLV was also rather low.

Nevertheless these data to a certain degree confirm the role of BLV in the etiology of bovine leukemia and the possibility of a horizontal route of BLV transmission from leukemic to healthy cattle.

These results also show that the appearance of typical signs of bovine leukemia (hematological and histological) precede the development of oncornavirus infection tested by the methods of molecular-biological and immunological assay (appearance of BLV structures in leukocytes and cells of lymph nodes, spleen; appearance of BLV antigen and antibody to BLV in serum). But it is necessary to point out that only small per cent of cattle — virus carriers and cattle with antibody became ill with hematological and histological expression of hemoblastosis.

Miller *et al.* (1972), Olson *et al.* (1975) showed that development of lymphocytosis in experimental cattle, inoculated with culture medium, was preceded by the appearance of C-type particles in lymphocytes, tested with electron microscopy. Van Der Maaten (1974) also demonstrated that some cattle, experimentally inoculated with BLV-containing culture medium had C-type particles in leukocytes (as was shown by electron microscopy) and antibody to BLV in serum (IDT). Although none of the experimentally inoculated animals had clinical nor hematological signs of bovine leukemia.

Some cattle had a subleukemic tendency to disease. They did not show any signs of disease during several years. In some cases the appearing signs of disease, for example hematological changes, regressed and only histological investigations of some of these animals, killed for diagnostic purpose revealed the signs of bovine leukemia. This is in accordance with bovine leukemia transmission experiment on cattle inoculated with cells of lymphosarcoma (Olson *et al.*, 1973).

These data confirm the supposition that bovine leukemia transmission experiments can give results only in several years. This is in accordance with the revealing of the spontaneous bovine leukemia in cattle of 5-7 years.

Some negative results can be explained not only by low leukogenicity of purified BLV but also an insufficiently long period (only 5 years)

of investigation of experimentally inoculated animals.

The possibility of bovine leukemia transmission to calves and sheep with milk was supposed by many investigators. First of all this supposition connects with revealing of C-type particles in milk of leukemic cattle (Dutcher and Larkin, 1964; Nazerian *et al.*, 1968; Schulze *et al.*, 1966; Rampichini *et al.*, 1975 etc.)

Later these theoretical premises received the first experimental confirmation in transmission experiments on calves (Weinhold and Straub, 1968; Straub, 1969, 1971, 1974; Hinze and Hull, 1976; Doronin *et al.*, 1977; Shickov *et al.*, 1976). McClure *et al.* (1974) demonstrated the development of leukosis in chimpanzee, fed with milk of leukemic cattle. But interpretation of these results is very difficult, because experimental animals were not investigated with virological, immunological methods and BLV also was not isolated from them.

The significance of all the abovementioned experiments on calves and monkeys is also reduced because the milk used for experimental feeding was not investigated the presence of BLV.

Our results obtained by the use of molecular-biological and immunological methods demonstrate the presence of BLV in milk and BLV reproduction in cells presenting in milk of leukemic cattle.

The reverse transcriptase activity of BLV structures of 1.14-1.18 g/ml density, isolated from milk and also from short term milk leukocyte culture was rather low. Perhaps it is connected not only with conditions of purification of BLV but more with the presence of inhibitors of RNA-dependent DNA-ase in milk (McCormick *et al.*, 1974; Furmanski *et al.*, 1976; Sanner, 1976).

It was shown also that milk of healthy and leukemic cattle contains epithelial, myoepithelial cells, foamy cells, histiocytes, macrophages, neutrophils, eosinophiles, lymphocytes and also free nuclei. The amount of blood cells was about 36.000-40.000/ml of milk (25 % of the total amount). The most of these cells were alive as demonstrated by vital staining with tripan-blue and by using a precursor of DNA-synthesis ^3H -thymidine.

It was shown that short term boiling of milk disturb the viability of cells in milk and their ability to produce BLV.

It was also demonstrated that oncornavirus infection tested in cells presenting in milk was the same and connected with oncornavirus infection revealed in blood leukocytes. So, the supposition was made that reproduction of BLV takes place in leukocytes presenting in milk of leukemic cattle.

The abovementioned data on the investigation of leukemic cattle milk and positive results of bovine leukemia transmission experiment by feeding calves with leukemic cow milk confirm the possibility of BLV transmission from leukemic to healthy cattle by feeding with raw milk.

Summary

The results of five years studies on the possibility of experimental induction of bovine leukemia with purified BLV and different BLV-containing materials demonstrated that inoculation of calves with purified BLV (structures of 1.14-1.17 g/ml density in 20-60 % sucrose gradient) can induce oncornavirus infection and bovine leukemia.

From 41 experimentally infected calves 9 showed evidence of bovine leukemia. 5 of these 9 animals were from a group of 22 calves inoculated with purified BLV. Diagnosis of bovine leukemia in these 9 animals was established by hematologic (3 animals) and histologic method (6 animals). BLV was isolated from leukocytes of all of them.

It was shown also that before the development of bovine leukemia the development of oncornavirus infection takes place. But only a small part of the animals with oncornavirus infection and presence of antibodies became ill with typical forms of hemoblastosis.

The investigation of the role of milk and blood cells in milk in bovine leukemia transmission was also carried out. The presence was demonstrated of oncornavirus structures antigenically identical with BLV in milk and reproduction of these structures in blood cells being present in milk. These results, and also the induction of bovine leukemia, in cattle by rearing with raw milk from cattle with leukemia, allow us to suppose the possible routes of bovine leukemia transmission in nature.

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