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BLV-p24 EXPRESSION IN BLV INFECTED CATTLE AND DETECTION OF BLV-p24 RECEPTORS IN CATTLE AFFLICTED WITH TUMOROUS LEUKOSIS *IN VIVO*

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Summary. — Noncultivated and short-term cultivated blood leukocytes of BLV-infected cattle both with and without tumorous leukosis have been investigated for BLV-p24 antigen expression and for their capacity to bind BLV-p24 antigen. In animals with persistent lymphocytosis using cell extracts of $3—6 \times 10^8$ non-short-term cultivated blood leukocytes, BLV-p24 antigen could be identified by means of competitive RIA. In cattle with tumorous leukosis, the antigen detection in non-short-term cultivated leukocytes may be masked by the presence of antigen-binding receptors. The capacity of p24 antigen binding is likely to be a phenotypical marker for the cells occurring in the tumour phase only.

Key words: *bovine leukaemia virus*; *cattle*; *antigen*; *receptors*

Introduction

The bovine leukaemia virus (BLV) is a lymphotropic RNA tumour virus; it can, after a certain latent period, induce lymphoproliferative diseases in form of persistent lymphocytosis (PL) and/or tumorous leukosis in cattle. BLV-infected cattle was found to produce antibodies against at least 4 viral structure antigens (Deshayes *et al.*, 1980). In the majority of animals this is frequently the only reaction to virus infection. The permanent production of BLV-specific antibodies can proceed only on the basis of a continuous or discontinuous presence of viral antigens *in vivo*. On the other hand, evidence for virus particles and viral antigens in blood lymphocytes of PL animals with and without tumorous leukosis has so far been provided only after *in vitro* cultivation (Stock and Ferrer, 1972; Baliga and Ferrer, 1977).

It is likely that *in vivo* antigen production is extremely low, being just at the detection limit of the procedure used, and/or that the antigens produced *in vivo* are present in a masked form, thus escaping direct immunological detection. The latter possibility is suggested by the following findings: 1. Plasma membranes from tumorous lymph nodes of BLV-infected cattle

bind the BLV antigens gp51 and p24 and should, consequently, have receptors for these BLV antigens. These receptors are not demonstrable in plasma membranes from lymph nodes of BLV-free cattle (Ristau *et al.*, 1986). 2. In extracts from cells of tumorous lymph nodes, the BLV antigens gp51 and p24 are not detectable by RIA, but application of these extracts to calves elicits production of antibodies against p24 (Ristau *et al.*, 1987).

To prove the existence of BLV receptors so far we have used exclusively samples from tumorous lymph nodes. In the present investigation, we looked for the presence of p24 receptors in blood leukocytes of BLV-infected cattle with persisting lymphocytosis as well as with tumorous leukosis; BLV-free cattle was used as control. Furthermore, we investigated whether there is any relationship between the presence of receptors for p24 and the detectability of p24 in the blood leukocytes of BLV infected cattle.

Materials and Methods

Animals. Group I: 16 BLV-free cows from a leukosis-free dairy farm. At the time of the investigation, the sera contained no antibodies against the BLV p24 and gp51 in the RIA. Group II: 26 BLV-infected cows without (n = 4) or with persistent lymphocytosis (n = 22; $9-52 \times 10^3$ leukocytes/µl blood) were investigated. The sera of these cows contained antibodies against the BLV antigens gp51 and p24 in the RIA. Group III: 19 BLV-infected cows with tumorous leukosis in form of multiple lymph node tumours (lymphadenosis, reticulosis mixed form, lymphosarcomatosis, and reticulosarcomatosis) were investigated. The sera of this cattle revealed antibodies against the BLV antigens gp51 and p24. Group IV: 9 BLV-free calves aged 6 months were immunized 3 times at biweekly intervals with a BLV gp51 preparation (Burkhardt *et al.*, 1988), applying 300 µg gp51 in each inoculation dose.

Cells. BL-3 cells: the tumour cell line BL-3 was kindly donated by G. H. Theilen (Davis, California): it stemmed from a cow with sporadic leukosis. Cells were multiplied in RPMI-1640 medium containing 10 % foetal calf serum. For testing the presence of receptors for p24, the BL-3 cells were treated in the same way as were blood leukocytes for radioimmunoassay.

Isolation of leukocytes. The leukocytes were isolated from cooled, heparinized bovine blood following distilled water shock (Weinhold, 1965). The cell sediments were washed twice with Eagle's Minimal Essential Medium (E-MEM) and sedimented by a washing procedure at 1500 rev/min (K 24-Janetzki) for 10 min at 4 °C. The number of dead cells was determined by the trypan blue exclusion method. Further were treated only those samples showing not more than 20 % dead cells.

Short-term cultivation and leukocyte extraction. Cultivation was performed with a concentration of $1-5 \times 10^7$ cell/ml in E-MEM in the presence of 10 %neonatal calf serum (SIFIN), 100 IU penicillin/ml, 0.1 g streptomycin/ml for 20 hr at 37 °C. To prepare LPS (lipopolysaccharide) stimulated leukocytes, 0.05 mg/ml LPS was added to the culture medium. After cultivation, cells were recovered from the culture flasks and the survival rate determined (trypan blue exclusion method, see above), which amounted to 75 % on the average. Subsequently, extraction buffer (0.02 mol/l Tris/HCl; 0.1 mol/l NaCl; 0.01 mol/l EDTA; 0.5 % NP 40; 0.2 % sodium deoxycholate; 2 mM Phenylmethansulphonylfluorid; pH 7.5) was added as described by Kenyon *et al.* (1981) and incubated at 25 °C for 20 min; thereafter the cells were frozen and thawed three times. Subsequently, the samples were centrifuged at 3000 rev/min (K 70-Janetzki) at 4 °C for 10 min, and the supernatant used for competitive radioimmunoassay (RIA).

Radioimmunoassays. 1. *BLV-gp51 and -p24 antibody detection in RIA.* Bovine sera were mixed in decreasing dilution with 1 ng ^{125}I gp51 (about 10 000 – 20 000 cpm) in a final volume of 400 µl with RIA buffer (0.05 mol/l PBS; 0.5 % HSA; 0.02 % NaN_3; 0.02 % Triton X-100) and incubated at 4 °C for 48 hr. After the incubation the immune complex was precipitated with rabbit anti-cattle immunoglobulin (in 200 µl RIA buffer). After another incubation (24 hr at 4 °C) the complex was washed with RIA washing buffer (0.05 mol/l PBS; 0.02 % NaN_3; 0.2 % Triton X-100), pelletted at 3000 rev/min (K 70-Janetzki centrifuge) for 30 min at 4 °C and, after decanting the washing solution, the bound radioactivity (pellet) was measured in a gamma counter.

Table 1. BLV-p24 antigen detection in noncultivated blood leukocytes from cattle with and without tumorous leukosis according to cell number

Animal groups	Leukocytes $\times 10^{-3}/\mu l$ blood	Antibodies p24	Antibodies gp51	Cell number $\times 10^{-8}$/probe	BLV-24 antigen detection in lymphocytes: noncultivated	BLV-24 antigen detection in lymphocytes: short-term cultivated
BLV-free	4—8	—	—	0.12 2.0	0/16[1])	0/16
BLV-infected without tumorous leukosis	36—52	+	+	3 —6	9/9	n.d.
				2	5/9	9/9
				1	2/9	9/9
				0.5	0/9	7/9
				0.25	0/9	5/9
				0.12	0/9	5/9
BLV-infected with tumorous leukosis	4—30	+	+	0.9 —3	positive 0/19 suspicious 3/19	positive 4/17 suspicious 6/17

[1]) Number of cell extracts with positive reactions/total number of examined cell extracts
n.d. — not determined

2. *BLV-p24 antigen detection.* Competitive BLV-p24 RIA was performed according to the method of Schmerr *et al.* (1980). The supernatants obtained from the short-term cultivated leukocytes after extraction and centrifugation were tested in a volume range of 25—200 µl (equivalent to 1×10^7 — 2×10^8 cells) for the presence of BLV-p24. To the supernatants were added 1 ng ^{125}I p24 (about 20 000 cpm) and a p24 standard cattle serum in a total volume of 400 µl RIA buffer. The reference value (B_0) contains the standard serum and 1 ng ^{125}Ip24 without added cell extract. All further steps were carried out as described above. The assay was calibrated with the same BLV-p24 antigen as used for radioactive labelling, and kindly donated by Dr. D. Portetelle (Faculté des Sciences Agronomiques de l'État, Gembloux — Belgique). In our RIA, the antigen detection limit was roughly 0.2 ng p24, which was equivalent to an inhibition of 18 %. The width of variation of the assay (B_0 — without added cell extract) is 10 % with m = 36 for 2 s 10 %. With added negative control extracts the width of variation is 12 % with n = 12 for 2 s, and 18 % for 3 s. It was established there from that all animals whose blood leukocyte extracts displayed less inhibition than 8 % are negative, and inhibition of 9—17 % was classified as suspicious region. From an inhibition of 18 % onward in the competitive RIA the results were considered positive.

BLV-p24 receptor detection. For detection of binding sites radioactively labelled p24 antigen with cell extracts, the same assay as described under point 2 was used. The additional binding of ^{125}Ip24 to an adjusted, radioactively labelled antigen-antibody complex was measured (B_0 value = 100 %). An additional binding exists when at least 118 % ^{125}Ip24 antigen becomes bound. The suspicious region is 117—108 %, and no additional binding exists when less than 108 % ^{125}Ip24 antigen is bound.

Results

In the short-term cultivated leukocytes from cattle with persisting lymphocytosis, using competitive RIAs, BLV-p24 was demonstrated in the extracts in each case if the cell number was at least 1×10^8 (Table 1). With the non-short-term cultivated leukocytes, the 3- to 6-fold amount of cells had to be

Table 2. Binding of ^{125}Ip24 antigen to cell extracts of noncultivated blood leukocytes from cattle with and without tumorous leukosis

Cattle	Leukocytes × 10^{-3} µl blood	Antibodies p24	Antibodies gp51	Binding of ^{125}Ip24 to cell extracts of noncultivated lymph	Cell number × 10^{-7}
GROUP I:					
BLV-free	4–8	–	–	0/16[1])	15–16
(LSP stimulated)	4–8	–	–	0/2	15–16
BL-3 cells		–	–	0/1	10
GROUP II:					
BLV-infected	4–8	+	+	0/4	1–20
(animals with PL)	9–16	+	+	0/10	10
	32–53	+	+	0/12	3–60
GROUP III:					
BLV-infected with tumorous leukosis	4–30	+	+	positive: 3/19 suspicious: 5/19	10–50
GROUP IV:					
BLV-free (immunized)	8–12	+	+	0/9	10–15

1) Number of cell extracts with positive reactions/total number of examined cell extracts

used in order to determine the presence of p24 antigen. In the group of 19 BLV infected cattle with tumorous leukosis we found, prior to cultivation of the leukocytes, suspicious antigen proofs in 3 animals; after the cultivation, 6 suspicious and, in another 4 animals, definitely positive p24 antigen proofs were obtained (Table 1). Table 2 exhibited cattle with ^{125}Ip24 binding capacity to cell extracts of leukocytes. We examinated non-short-term cultivated leukocytes obtained from healthy cattle and from cattle at various stages of BLV infection. In leukocyte extracts of BLV-free animals, no receptors for

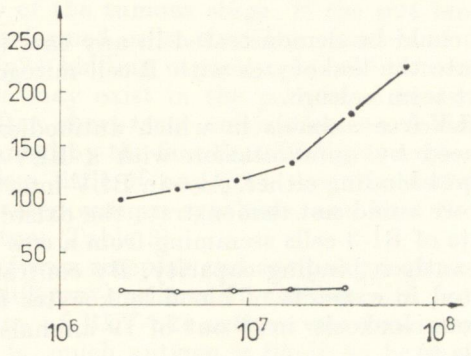

Fig. 1.
Binding of ^{125}Ip24 antigen to cell extract from blood leukocytes of tumorous cattle
●——● cell extract noncultivated blood leukocytes
○——○ cell extract short-term cultivated blood leukocytes
Abscissa: leukocyte number; ordinate: ^{125}Ip24 binding (%).

Table 3. Presence of antigen and receptors for BLV-p24 in the blood leukocytes of cattle with tumorous leukosis

Cattle No.	Detection of ^{125}Ip24-BLV antigen in cell extracts of		Binding of ^{125}Ip24 to cell extracts of	
	noncultivated cells	short-term cultivated leukocytes	noncultivated cells	short-term cultivated leukocytes
2	−	−	−	−
4	−	−	−	−
5	−	−	+	−
10	−	(+)	−	−
11	−	(+)	−	−
13	−	(+)	−	−
14	−	+	−	−
19	−	+	−	−
7	(+)	(+)	−	−
8	(+)	(+)	−	−
9	(+)	(+)	−	−
6	−	−	(+)	−
12	−	−	+	−
1	−	−	+	−
3	−	−	+	−
15	−	+	(+)	−
17	−	n.d.	(+)	n.d.
16	−	n.d.	(+)	n.d.
18	−	+	(+)	−

n.d. — not determined
− — negative
+ — positive
(+) — suspicious

p24 could be demonstrated in any case (Table 2), even when trying to stimulate the leukocytes with B-cell mitogen LPS (lipopolysaccharide) in the short-term culture.

BLV-free animals in which antibodies against gp51 and p24 had been induced by immunization with a BLV-gp51/p24 preparation, showed no ^{125}Ip24 binding either. Also in BLV-infected animals, either with or without PL, we could not demonstrate the existence of receptors. Likewise, cell extracts of B1-3 cells stemming from a cow with sporadic leukosis revealed no p24-antigen binding capacity. By contrast, p24 receptors could be demonstrated in extracts of blood leukocytes from BLV-infected cattle with tumorous leukosis in 8 out of 19 animals (3 animals positive, 5 suspicious, Table 2).

Fig. 1 shows a representative example for the ^{125}Ip24 binding capacity of the extract of noncultivated blood leukocytes from a cow with tumorous leukosis, with a leukocyte count of 1.2×10^5 cells per µl blood. After 24 hr of cell cultivation, however, complete abolition of the binding capacity of ^{125}Ip24 to the receptors was observed.

Discussion

The results in Table 1 show clearly that, by using sensitive methods, expression of the BLV-p24 antigen can be detected also in noncultivated blood leukocytes of BLV-infected cattle with PL, and lend support to the results of Nötzel *et al.* (1982) and Cuong (1984) who demonstrated the expression of BLV-RNA sequences in lymphocytes by means of *in situ* hybridization with BLV-^{3}H-cDNA. The use of too low cell quantities probably is the reason why various authors (Gupta and Ferrer, 1981; Baliga and Ferrer, 1977; Kenyon and Piper, 1977; Kenyon *et al.*, 1981) were unable to prove the existence of antigens in non-cultivated leukocytes using RIA.

In an earlier paper, Ristau *et al.* (1986) have shown that plasma membranes from tumorous lymph nodes of BLV-infected cattle have receptors for the BLV antigens gp51 and p24. To prove receptors for BLV-p24, we determined the ^{125}Ip24 binding capacity to cell extracts of non-short-term cultivated leukocytes. Receptors of BLV-p24 could be demonstrated in extracts of blood leukocytes from BLV-infected cattle with tumorous leukosis.

The binding capacity of antigens to cell extracts is likely to be a phenotypical marker (receptor) for the cells occurring only in the tumour phase. McGrath *et al.* (1978) and McGrath and Weissman (1979) have demonstrated in murine leukosis that T-cell lymphoma cells express surface receptors that are specific for lymphoma-inducing virus, and did not occur in virus-infected preleukaemic thymocytes. Not in all tumour-bearing animals we could demonstrate binding of ^{125}Ip24. According to Marshak *et al.* (1962), discharge of neoplastic cells into the blood occurs only in about one-third of the cows with tumorous leukosis. Kettmann *et al.* (1980) found polyclonal BLV provirus integration sites in peripheral blood leukocytes in tumour-bearing animals at a varying quantitative ratio. Monoclonal provirus integration sites are characteristic of the tumour stage. If the p24 binding is characteristic of malignant transformed cells, then the number of these cells in blood determines the ^{125}Ip24 binding capacity. In addition to the malignant transformed cells, there may exist in the peripheral blood also BLV-antigen expressing cells. After short-term cultivation we found no free receptors. This might be explained by the fact that BLV-p24 expressing lymphocytes, which *in vivo* express little if any p24, are stimulated by the short-term cultivation to increased antigen synthesis, and that the expressed p24 is bound to the receptors (Table 3).

Table 3 shows the correlation between receptor and antigen proofs among cattle with tumorous leukosis in different variants. In animals with positive receptor findings prior to cultivation, no BLV-p24 antigen was demonstrated after short-term cultivation, that is, much antigen is likely to be required

for saturation of the receptors. With suspicious receptor findings prior to cultivation, the tumour-bearing animals exhibit a broad reaction spectrum following short-term cultivation, as reflected by negative up to positive p24 antigen findings. In the latter case, an excessive antigen production should occur, which still leads to competition for receptor saturation. Preparation of the cell extracts did not allow to distinguish as to where the receptors were localized — inside or outside of the cell membrane.

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