

DETECTION OF BOVINE LEUKAEMIA VIRUS RNA SEQUENCES IN NON-CULTIVATED PERIPHERAL LYMPHOCYTES BY IN SITU HYBRIDIZATION WITH ³H-LABELLED VIRAL cDNA

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Summary. — Bovine leukaemia virus (BLV) in circulating lymphatic cells of the blood is repressed and has to be activated for its expression. After in situ hybridization of BLV-³HcDNA with a specific activity of 3.9×10^7 dpm/ μ g to noncultivated blood cells from 5 leukotic cattle, in 4 of these animals from 0.96% to 1.5% lymphocytes were found to contain BLV-specific RNA sequences. Non-hybridized lymphocytes as well as calf thymus cells, used as controls, gave only 0.47% and 0.39% labelled cells, respectively. After short-term cultivation of white blood cells from these leukotic animals the percentage of labelled cells increased. During the short-term cultivation the number of hybridizing cells from one and the same animal and from all animals, when compared with each other, varied to a high extent. The highest values of BLV-RNA sequences-containing cells from all 5 animals at different cultivation times were within the range of from 2.4 to 16.2%.

Key words: bovine leukaemia virus; persistent lymphocytosis; peripheral blood lymphocytes; viral cDNA in situ hybridization

Introduction

Enzootic bovine leukosis (EBL), a lymphoproliferative disease of cattle is caused by bovine leukaemia virus (BLV) (Miller *et al.*, 1969; Wittmann, 1970; Callahan *et al.*, 1976; Kettmann *et al.*, 1976). After experimental infection with this exogenous retrovirus (Kettmann *et al.*, 1976), a humoral antibody response to structural antigens of the virus rapidly occurs (Burny *et al.*, 1978; Mussgay and Kaaden, 1978). In the following course of the disease persistent lymphocytosis (PL) or tumour development or both may take place.

PL is characterized by a permanent increase in the number of circulating lymphoid cells of the blood (Götze *et al.*, 1953; Bendixen, 1963; Tolle, 1964). A variable percentage of PL animals develop lymphoid tumours (Schmidt *et al.*, 1970; Ferrer *et al.*, 1979).

In the genomic DNA of lymphatic cells of the blood and of lymphoid tumours, BLV provirus DNA can be demonstrated (Kettmann *et al.*, 1976; 1979, 1980a). On the other hand neither structural antigens (Miller *et al.*, 1969; Ferrer *et al.*, 1971, 1972; Stock and Ferrer, 1972; Baliga and Ferrer 1977) nor BLV-RNA were detected in circulating lymphatic cells from PL animals after hybridization in solution (Kettmann *et al.*, 1980a, b). This led to the conclusion that BLV in circulating lymphatic cells is repressed and has to undergo activation to become expressed (Miller *et al.*, 1969; Baliga and Ferrer, 1977). However, the low sensitivity of the methods employed did not allow to give a clear-cut answer.

Infections with visna virus could be demonstrated by a very sensitive *in situ* hybridization method, which revealed that lymphocytes from infected animals harbour viral RNA, despite the fact that viral proteins were not found (Brahic and Haase, 1978). Therefore, we tried to detect BLV-specific RNA sequences in circulating lymphatic cells of the blood from cattle with PL by an *in situ* hybridization method with BLV-³HcDNA and looked for the influence of short-term cultivation on the expression of viral RNA in these cells.

Materials and Methods

White blood cells (WBC). Peripheral blood was collected by puncturing the jugular vein. WBC were isolated by the method described by Weinhold (1965) and cultivated for 3, 24, 48, 68 and 116 hr in Eagle's minimal essential medium containing 200 µg/ml streptomycin, 200 µg/ml penicillin, 10% foetal calf serum and 50 µg/ml lipopolysaccharide. At the intervals indicated, cell samples were washed with phosphate buffered saline, smeared on glass slides and air-dried. The supernatant was used for testing reverse transcriptase activity (Roessler *et al.*, 1980).

Synthesis of BLV-³HcDNA. Virus-specific cDNA was synthesized in a standard endogenous reaction mixture (0.5 ml) containing purified BLV from infected foetal lamb kidney cells (van der Maaten cell line) with a total viral protein amount of 1.5-2 mg; 0.05 mol/l Tris-hydrochloride, pH 8.3; 0.005 mol/l MgCl₂; 0.06 mol/l NaCl; 0.01% Triton X-100; 0.03 mol/l dithiothreitol, 1 mmol/l of each ribonucleoside triphosphate (ATP, CTP, GTP, UTP); unlabelled deoxynucleoside triphosphates (3 × 10⁻⁵ mol/l dATP, 3 × 10⁻⁵ mol/l dCTP, 1 mmol/l dGTP); 37 MBq ³H-dATP (78 MBq/mmol); 37 MBq ³H-dCTP (170 MBq/mmol); 74 MBq ³H-dTTP (555 MBq/mmol); 10% polyethylene glycol 6000; 50 µg Actinomycin D; and 1 mg deoxyribonuclease-digested calf thymus DNA. These mixtures were incubated for 18 hr at 37° C and the reaction was terminated by adding 0.01 mol/l EDTA, sodium dodecyl sulfate to 0.5%, and pronase (500 µg/ml). The reaction mixtures were further incubated for 30 min at 37° C, extracted twice with 1 volume of phenol-cresol (7 : 1) and 1 volume chloroform-isoamylalcohol (24 : 1), and precipitated with ethanol. The total nucleic acid product was then separated from free nucleoside triphosphates by chromatography on Sephadex G-50. Hydrolysis of RNA from RNA-DNA hybrids was performed by incubation in 0.4 mol/l NaOH for 4 hr at 37° C followed by centrifugation in alkaline linear sucrose gradient (5-20%) in 0.1 mol/l NaOH, 0.9 mol/l NaCl, 0.002 mol/l EDTA. Those fractions containing cDNA molecules with an average length of 1000 to 1500 nucleotides were pooled, neutralized, and sonicated for hybridization.

In situ hybridization. Fixation and preparation of smears and *in situ* hybridization were done as described by Brahic and Haase (1978) with few modifications. Duration of treatment of WBC with proteinase K (1 µg/ml) was shortened from 15 min to 5 min. This step is a very critical one because after various times of short-term cultivation the cells become differently sensitive to this treatment. The hybridization mixtures (total volume of 6 µl) contained 10 mmol/l Tris-HCl pH 7.4; 1 mmol/l EDTA; 600 mmol/l NaCl; 0.2 g/l Ficoll, 1 mg/ml bovine serum albumin; 0.2 g/l polyvinylpyrrolidone, 100 µg/ml poly(A); 100 µg/ml calf thymus DNA (sonicated); 1 mg/ml transfer RNA; 1 mg/ml ribosomal RNA; 15000 dpm BLV-³HcDNA and 50% formamide. After

denaturation at 100° C for 30 sec, the mixture was incubated for 60 min at 50° C and then applied to the treated cells. Hybridization was allowed to occur at 45° C from 44 to 60 hr. Unspecific hybrids were removed by incubation in $2 \times$ SSC (300 mmol/l NaCl, 30 mol/l sodium citrate) at 56° C for 60 min (Kaufmann *et al.*, 1979).

Autoradiography. The smears were dried in ethanol containing 300 mmol/l ammonium acetate, air-dried and then tipped in Orwo emulsion K 6, diluted 1 : 0.8 with water-ethanol-glycerin (3.6 : 0.24 : 0.16) and after drying exposed at 4° C for two weeks. The smears were developed for 3 min in Orwo developer MH-28 (1 : 3), fixed in Orwo A 324 for 2 min, washed and stained according to Giemsa.

Evaluation of the autoradiographs. The autoradiographs were evaluated in transmitted light in a Fluoval (VEB Carl Zeiss Jena) microscope with oil immersion objective. Photographs were taken at a 320-fold magnification. For each sample the numbers of granules were counted a) in the cell free areas to determine the background value, and b) within the cells. Per 100 μm^2 cell-free areas, the numbers of granules never exceeded 4. Cells with 8 and more granules were evaluated as positive. To correct the number of granules within the positive cells, we subtracted the number of granules which were counted in the cell-free areas, taking into account the size of the corresponding areas. The biological background of the method will be described along with the results.

Results

Direct demonstration of viral RNA is possible by means of in situ hybridization and with highly labelled viral cDNA (Pardue and Gall, 1975). We synthesized endogenously BLV- ^3Hc DNA with a specific activity of 3.9×10^7 dpm/ μg , using detergent-disrupted BLV, and hybridized this labelled cDNA with WBC from leukotic cattle in situ. One control consisted in autoradiography of peripheral WBC which were not hybridized with BVL- ^3Hc DNA. Among 2,000 cells the percentage of cells with 7.5 granules (average grain count per cell) amounted to 0.47%. The highest value observed did not exceed 8 granules per cell. Another control was to count the granules within the cell-free areas. Here, the number of granules never exceeded 4 per 100 μm^2 . This value did not increase even when hybridization was carried out. Based on these data we determined the confidence interval of a specific formation of granules to be 8 and more per cell. Finally, a third type of control consisted in hybridization of BLV- ^3DNA to calf thymus cells, to

Table 1. BLV-c DNA hybridization to peripheral lymphocytes from cattle before and after short-term cultivation

Animal No.	WBC per μl blood	RTA*	BLV- ^3Hc DNA hybridized to					
			noncultivated WBC			cultivated WBC**		
			Total	Labelled %	Grains/cell	Total	Labelled %***	Grains/cell
55113	57 200	ND	2054	1.1	12.1	274	7.7	10.8
45107	40 700	+	2180	1.5	16.0	664	2.4	9.5
56369	24 200	+	2062	0.48	14.4	222	16.2	15.4
55265	10 400	ND	2013	1.24	9.9	661	12.6	11.9
00002	10 000	+	2180	0.96	15.5	660	4.2	17.8

* Reverse transcriptase activity: ND = not done.

** WBC were cultivated for 3 (No. 55265), 24 (Nos 55113, 45107, 00002) or 68 (No. 56369) hr.

*** Maximal percentage obtained during the total cultivation period.

which before this procedure pre-hybridization with poly A, rRNA, tRNA and calf thymus DNA had been applied. As calf thymus cells (T lymphocytes) cannot be infected with BLV (Paul *et al.*, 1977a), an eventual positive in situ hybridization would indicate the presence of non-viral DNA sequences within the cDNA probe used. The percentage of calf thymus cells having 8 or more granules was up to 0.39, thus corresponding to the above-mentioned percentage of non-hybridized control cells (0.47) giving a false-positive answer. From these data we concluded that a percentage of 0.5 labelled cells represented the background value of our method.

The biological specificity of our BLV-³HcDNA preparation was checked by in situ hybridization to BLV-infected FLK cells (van der Maaten cell line) which, within their chromosomal DNA, contain the BLV proviral DNA (Kettmann *et al.*, 1978, 1980a). According to Astier *et al.*, (1978), the van der Maaten cells are producing about 3000 viral 35 S RNA copies. However, Kettmann *et al.* (1980a) reported the detection of not more than 30 viral RNA copies under his conditions. Possibly, BLV production in the van der

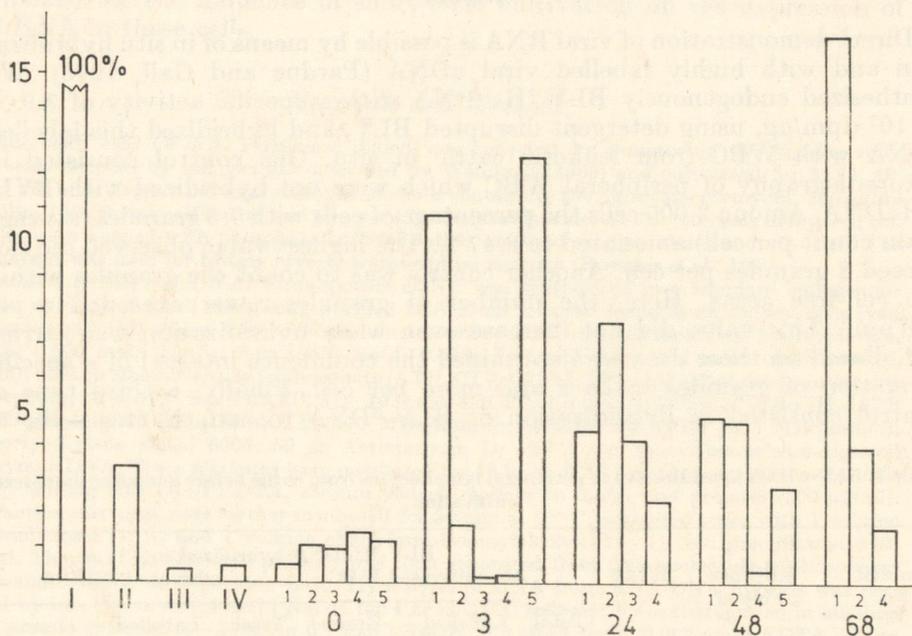


Fig. 3.

Estimation by in situ hybridization with BLV-³HcDNA of BLV-RNA positive WBC
 Abscissa: 0-68 = hr of WBC cultivation; I-IV = controls; in situ hybridization with BLV-³HcDNA to FLK cells (I); FLK cells treated for 60 min with 1 mg/ml ribonuclease (II); calf thymus cells (III); and WBC from leukotic cattle processed for in situ hybridization without BLV-³HcDNA to determine nonspecific labelling of cells (IV).

Ordinate: % BLV-positive cells

Columns 1-5: WBC from leukotic cattle (animals Nos 56369, 55113, 00002, 45107 and 55265, respectively)

Maaten cells varies within broad limits. After in situ hybridization of our van der Maaten cells with BLV-³HcDNA we found that all cells contained on the average 29.2 granules per cell (Figs 1 and 2). The number of granules, however, varied between 8 and 101 per cell. When the cells were treated with ribonuclease prior to the hybridization procedure, the percentage of hybridization+positive cells was lowered from 100 to 3.4, and the average number of granules within the positive cells was lowered to 11.8. We believe that the degradation of RNA was incomplete so that we could not obtain the zero value of 0.5% labelled cells. These control experiments clearly showed that the BLV-producing van der Maaten cells had been labelled specifically with the BLV-³HcDNA probe.

We then performed in situ hybridization experiments with non-cultivated peripheral WBC from 5 animals, 3 of which were suffering from pronounced

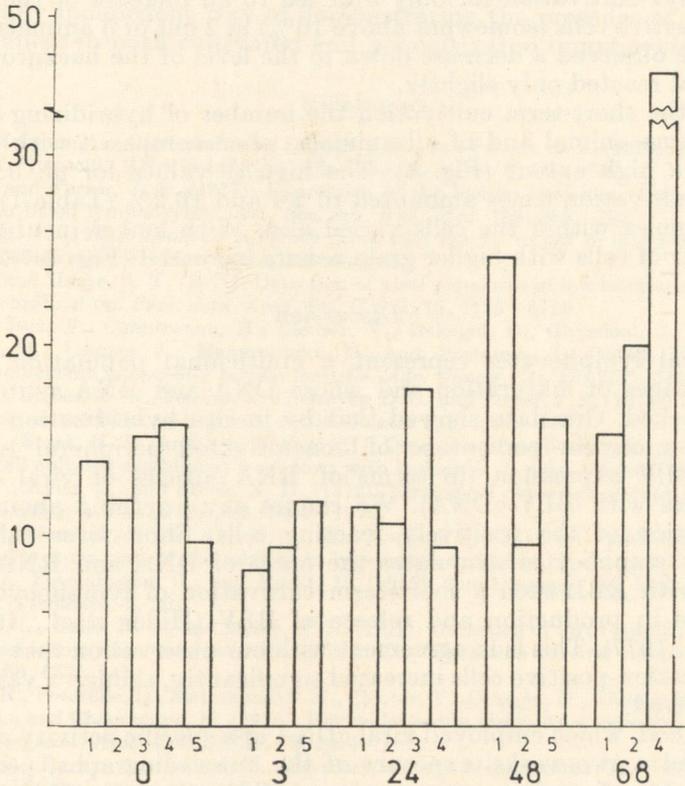


Fig. 4.

In situ hybridization of BLV-³HcDNA to WBC from leukotic cattle
 Abscissa: time of cultivation in hr; ordinate: average grain count per cell corrected for cell-free background value
 Columns 1-5 as in Fig. 3.

persistent lymphocytosis and 2 others showed a weak enhancement of WBC (Göttingen key). As can be seen from Table 1 and Fig. 2, only in one animal the percentage of labelled cells was around 0.48 and hence within the background value. However, from data reported by Roessler *et al.* (1980) it was known that this animal reacted positively with the reverse transcriptase test after short-term cultivation of its lymphocytes. Therefore, it is justified to conclude that all 5 animals were BLV-infected.

The number of granules within the cells varied from 9.8 to 16.0 per cell (Fig. 3). Taking into account the size of the cells, the variation becomes less significant, and values from 16.0 to 19.2 granules per 100 μm^2 cell area were obtained. It is noteworthy that the number of granules dropped to 11.1 per 100 μm^2 of ribonuclease-treated van der Maaten cells and calf thymus cells. By short-term cultivation of peripheral WBC (from 3 to 116 hr), the percentage of cells able to hybridize with BLV- $^3\text{HcDNA}$ could be increased significantly. Cultivation for only 3 hr led to an increase of the percentage of BLV-positive cells (somewhat above 10%) in 2 out of 5 animals. In 2 other animals we observed a decrease down to the level of the background values. One animal reacted only slightly.

During the short-term cultivation the number of hybridizing cells of one and the same animal and of all animals, when compared with each other, varied to a high extent (Fig. 4). The highest values for all 5 animals at different cultivation times amounted to 2.4 and 16.2% (Table 1). The number of granules within the cells varied also. With longer incubation times, the number of cells with higher grain counts increased (Figs 5-7).

Discussion

Peripheral lymphocytes represent a multiclonal population of cells in different stages of maturation and whose DNA and RNA synthesis is completely blocked. Our data showed that by *in situ* hybridization with BLV- $^3\text{HcDNA}$ a certain percentage of noncultivated peripheral lymphocytes revealed BLV expression (in terms of RNA species of viral origin able to hybridize with BLV-cDNA). We cannot say anything about the stage of maturation of the positively reacting cells. Short-term cultivation of peripheral lymphocytes stimulates the onset of DNA and RNA synthesis; in cattle with EBL such a short-term cultivation of lymphocytes leads to an increase in production and release of BLV (Miller *et al.*, 1969; Baliga and Ferrer, 1977). This is in agreement with our observation that the number of hybridization-positive cells increased significantly, though a value of 100% never occurred.

Our method, which employed viral cDNA of a specific activity of 3.9×10^7 dpm/ μg and a two-weeks' exposure of the autoradiographs, permitted the demonstration of nucleic acid sequences within the cells. We assume that these sequences belong to viral RNA species (genomic viral RNA and viral mRNA) because of the ribonuclease sensitivity of the granules, and that these viral RNAs are transcription products from the integrated provirus DNA.

Kettmann *et al.* (1978) were able to demonstrate that not all lymphocytes in the stage of PL are BVL-infected. They concluded that this was due to the multiclonal origin of the cells. According to these authors only 2 provirus genomes are present in a diploid lymphocyte. Paul *et al.* (1977b), using electron microscopy, found after short-term cultivation (72 hr) virus particles in 23% of the lymphocytes. This percentage could be increased twice by stimulation with phytohaemagglutinin. Kettmann *et al.* (1980b) estimated the percentage of BLV provirus-containing cells by a hybridization technique in solution. The value of 25% reported by them is somewhat higher than in our experiments (1.7–16%). Different conditions of hybridization in solution and in situ and of cultivation might be responsible.

After the report by Roessler *et al.* (1980), who detected reverse transcriptase activity not only in cultivated but also in noncultivated lymphocytes from BLV-infected cattle by an endogenous test system, we now showed another methodically independent way of demonstrating the presence of BLV nucleic acid sequences in both cultivated and noncultivated lymphocytes.

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Explanation of Micrographs (Plates V—VII):

Fig. 1. BLV-³HcDNA-labelled FLK cells.

Fig. 2. BLV-³HcDNA-labelled FLK cells; dark field photograph:

Fig. 5. Circulating WBC from animal No. 55265 labelled after in situ hybridization to BLV-³HcDNA, before short-term cultivation. Cells with more than 7 granules per cell were regarded as positive.

Fig. 6. WBC from animal No. 55265 after 3-hr cultivation labelled with BLV-³HcDNA; in situ hybridization. Only cells with more than 7 granules per cell were counted.

Fig. 7. BLV-³HcDNA-labelled WBC after in situ hybridization following 48 hr of cultivation. Cells with very high counts of granules can be seen after this prolonged cultivation.