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TREATMENT OF BOVINE LEUKAEMIA VIRUS-INFECTED SHEEP WITH SURAMIN: AN ANIMAL MODEL FOR THE DEVELOPMENT OF ANTIRETROVIRAL COMPOUNDS

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Summary. — Bovine leukaemia virus (BLV) and the human T-cell leukaemia/lymphoma viruses I and II represent a specific group of type-C RNA tumour viruses characterized by the presence between the *env* gene and the 3'LTR of an "x" region or LOR frame, which codes for a protein that trans-activates the transcription of the viral genome. As BLV can also infect sheep and induces preB-cell specific tumours in these animals, we were interested in investigating whether suramin, a potent inhibitor of retrovirus-associated reverse transcriptase, may inhibit the *in vivo* multiplication of BLV in sheep. The sheep were infected with 4×10^7 leukocytes from a BLV-infected cow. The animals were maedi-visna virus-negative. Viral p24 antigen and reverse transcriptase appeared at 2 weeks and seroconversion occurred at 4 weeks after infection. Suramin was administered at 20 mg/kg/week from the 10th till the 16th week after infection. During the treatment period the expression of p24 antigen as well as the titre of anti-p24 and anti-gp51 antibodies were followed. Suramin treatment led to a significant, but transient, disappearance of p24 antigen and did not affect the titre of anti-p24 and anti-gp51 antibodies. The BLV-infected sheep may serve as a useful animal model for the investigation of retrovirus inhibitors and the evaluation of different therapeutic regimens.

Key words: *bovine leukaemia virus*; *p24 protein*; *reverse transcriptase*; *suramin*; *sheep*; *experimental therapy*

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Introduction

The discovery of HIV as the causative agent of AIDS has led to reevaluation of reverse transcriptase (RT) inhibitors, because (1) the virus belongs to the family of *Retroviridae*, genus *Lentivirinae*, and (2) the RT plays a key role in establishing infection and viral expression. Some of the RT inhibitors like suramin, HPA-23, phosphonoformate and azidothymidine have already been used for the clinical treatment of AIDS. Suramin and structurally related non-ionic polysulphonic dyes are effective competitive inhibitors of RT *in vitro* (De Clercq, 1979, 1986). The ID_{50}s of suramin, HPA-23 and azidothymidine 5′-triphosphate for the RT of BLV are 2.8, 8.0 and 0.17 μmol/l, resp. (Reimer *et al.*, 1989).

Usually, leukaemic mice serve as animal models for assessing the antiretroviral effect of chemical substances. We now introduce BLV-infected sheep as a useful model. Together with HTLV-I and HTLV-II (Wong-Staal and Gallo, 1985), BLV makes up a group of type-C retroviruses which:

- can be transmitted horizontally
- induce chronic leukaemia/leukosis in natural populations
- do not possess endogenously inherited proviral sequences
- do not have *onc* genes but code for one or more gene products which trans-activate viral RNA synthesis and replication
- are viraemic and thereby induce humoral and cell mediated immune reactions
- cause the formation of syncytia.

On the other hand, BLV, HTLV-I, HTLV-II and lentivirus infections have certain features in common (Haase, 1986), in that they show a long lasting latency period, chronic course of the disease and trans-activation or trans-regulation of transcription. Sheep can be experimentally infected with BLV (Wittmann *et al.*, 1971). The virus induces pre B-cell specific tumours (Levy *et al.*, 1987). Kenyon *et al.* (1981) reported BLV infection of sheep by using different virus sources, i.e. short-term cultivated lymphocytes from the peripheral blood of an infected cow, permanently BLV-infected lymphocyte cultures (NBC-13) and cell free supernatant of BLV-infected bat lung cells. Forty per cent of animals which were infected as newborn lambs developed lymphosarcomas between 15 and 24 months post infection (p.i.).

Materials and Methods

Animals. The 9 sheep were from one herd, they were 6 months old and female and did not have BLV-p24 antigen or anti-p24 antibody. They were also negative for maedi-visna virus (MVV) infection as determined by an agar gel immunodiffusion test (AGIDT). Two sheep, serving as control, were neither infected nor treated. Seven sheep obtained each 1 ml heparinized blood from one cow with BLV-induced persistent lymphocytosis (43,000 leukocytes/μl blood). The route of infection was intraperitoneal. The donor cow was positive in all BLV tests: p24, anti-p24, anti-gp51 (all determined by means of radioimmunoassay (RIA)) and RT. Three infected animals were subjected to therapy, and four infected animals served as control.

Therapy. Three sheep which had been consistently positive for p24 antigen were subjected to therapy at the tenth week p.i. They were given suramin (germanin, Bayer 205) intravenously at

a dose of 20 mg/kg/week for a period of 6 weeks. Four days after each injection blood samples were collected from all animals. The animals received 4.2 g (no. 604), 3.9 g (no. 672) and 3.6 g (no. 728) suramin.

Short-term cultivation of leukocytes. Leukocytes from 100 ml precooled heparinized blood were isolated according to Weinhold (1965), and cultured according to Larsen (1979) with slight modifications (using RPMI-1640 medium supplemented with 10 % neonatal calf serum and 20 μg phytohemagglutinin (PHA)/ml). Cultivation was for 24 hr at 37 °C. A longer cultivation period did not give an increased p24 synthesis in the BLV-infected sheep lymphocytes.

Reverse transcriptase estimation. RT was determined according to Rössler *et al.* (1980), using the supernatant of short-term cultivated leukocytes.

Detection of BLV p24 antigen. The p24 antigen content was estimated in lysates of short-term cultivated leukocytes by means of a competitive RIA test, according to Schmerr *et al.* (1980).

Detection of anti-p24 and anti-gp51 of BLV and antibodies to MVV. For the detection of the BLV antibodies we used the method described by Mammerickx *et al.* (1980). Detection of antibodies against MVV was accomplished by using the AGIDT described by Dawson *et al.* (1979).

Infection assay. Two to three healthy recipient sheep received 5—10 ml blood s.c. of the donor sheep. Four weeks later the ovine sera of the recipients were checked for BLV specific antibodies (AGIDT).

Results

Experimental design

Seven sheep, which were MVV-free, were infected with BLV-containing lymphocytes from cattle. The infection was followed for a period of 36 weeks. The serum of all animals was examined weekly for anti-p24 by RIA. For detection of virus we used short-term (24 hr) cultivated PHA-stimulated lymphocytes. RT activity was measured in the cell culture supernatant and p24 antigen in the cell lysate. The sensitivity of the p24 RIA test was such that it allowed to detect 0.2 ng per 10^7 cells. After it had been unequivocally established that BLV infection took place, 3 animals were treated with suramin. The treatment regimen for suramin was the same as that applied for patients suffering from trypanosomiasis, that is 20 mg/kg/week, administered intravenously for 6 weeks.

Table 1. Reverse transcriptase activity and p24 antigen in BLV-infected sheep

Sheep no.	BLV-inf.	p24 1—9 weeks p.i. pos./ total n/n	Reverse transcriptase activity (dpm per supernatant of 5 × 10^7 cells) weeks post infection						
			1	2	3	4	5	6	7
512	—	0/6	0	0	0	0	0	n.d.	n.d.
599	—	0/6	0	0	0	0	0	n.d.	n.d.
671	+	8/9	0	1.470	24.145	0	730	7.045	8.610
691	+	2/9	0	1.070	8.190	2.720	2.425	0	8.540
622	+	6/6	0	1.250	3.345	0	0	n.d.	n.d.
735	+	4/6	0	3.895	0	1.940	0	n.d.	n.d.
604	+	6/6	0	4.580	5.625	1.130	1.410	n.d.	n.d.
728	+	6/6	0	2.970	0	0	1.725	n.d.	n.d.
672	+	6/6	0	0	0	0	0	n.d.	n.d.

n.d.: not determined

The experiment comprised four periods:

1. the stage prior to virus infection
2. the incubation period
3. the treatment period
4. the post-treatment period.

Non-infectious stage of the animals

At 6 months of age, before infection with BLV, the sheep were analysed for the absence of BLV antigen and antibodies. When using the RIA for detecting anti-BLV-p24, or, when using, after short-term cultivation of lymphocytes, the RT test or the RIA for BLV p24, neither test gave a positive result. The absence of MVV antibodies was confirmed by means of AGIDT.

Experimental BLV infection

The 7 sheep were inoculated intraperitoneally with 1 ml blood from a cow with enzootic bovine leukosis (eBL) in the stage of persistent lymphocytosis having 4.3×10^4 leukocytes per µl blood. Thus, each animal received 4.3×10^7 leukocytes. Two animals, which were neither infected nor treated, served as controls. Within the first week p.i. there were no signs of infection, but as early as 2 weeks p.i. we found in the 7 infected animals p24 antigen in the lysate of the short-term cultured cells. The RT also became detectable

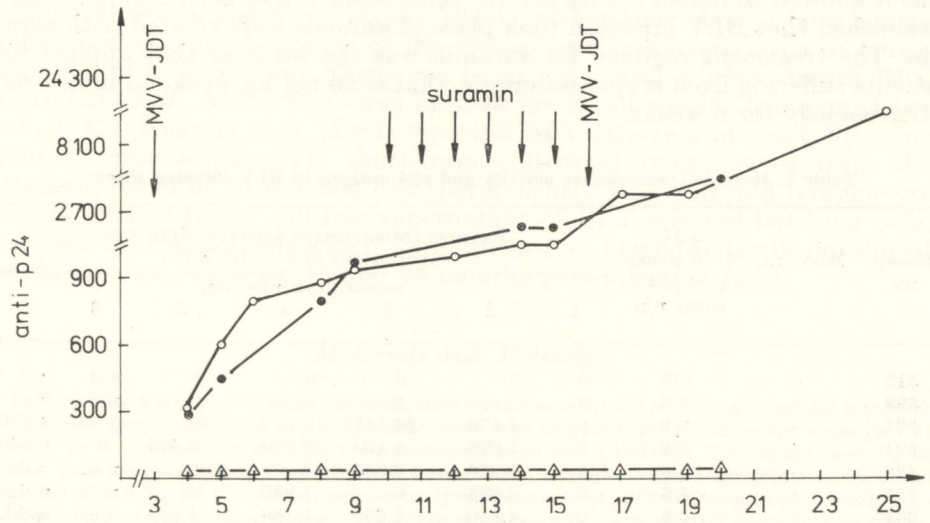

Fig. 1.

Anti-p24 antibody titre in sera of BLV-infected sheep with and without ●———● suramin treated: ○———○ nontreated, infected; △———△ noninfected Abscissa: weeks post-infection; ordinate: anti-p24 antibody titre (dilution reciprocals).

Table 2. Detection of p24 antigen in lymphocytes of BLV-infected and suramin-treated sheep

Animal no.	Pre-infection		Post-infection 1—9 weeks p.i.		Treatment p24 (weeks p.i.)						Post-treatment p24 (weeks p.i.)		
	RT	p24	RT	p24	10	11	12	13	14	15	16	32	36
control													
512	0	0	0/4	0/6	0	0	0	0	0	0	0	0	0
599	0	0	0/4	0/6	0	0	0	0	0	0	0	0	0
infected													
691	0	0	5/9	2/9	0	n.d.	n.d.	0	n.d.	0	0	0	0
671	0	0	5/9	8/9	(+)	n.d.	n.d.	+	+	+	+	+	+
622	0	0	2/4	6/6	+	n.d.	+	+	+	n.d.	(+)	+	+
735	0	0	1/4	4/6	+	n.d.	n.d.	+	+	0	0	0	0
infected and treated													
604	0	0	4/4	6/6	+	0	(+)	(+)	0	0	+	0	0
672	0	0	0/4	6/6	+	0	0	0	0	0	+	0	0
728	0	0	2/4	6/6	+	0	+	+	0	0	(+)	0	0

0 = negative; + = positive; (+) = doubtful; n.d. = not determined

in the supernatant of these cells, albeit less consistently Table 1 . After the 2nd week 6 out of 7 animals were RT-positive, after the 3rd week 4 out of 7, and after the 4th week 3 out of 7 animals. Only 3 of the 7 animals were consistently positive in the RT test between the 2 nd and 7th week p.i. One animal, though always positive for p24 antigen, never became RT positive. The RT activity ranged from 1,000 to 24,000 dpm per supernatant of 5×10^7 cells in 13 out of the 20 positive measurements (altogether 32 tests were performed). The number of BLV-infected and BLV-expressing cells fell below the RT-detectable threshold following suramin treatment. Therefore, determination of RT activity was discontinued. However, we continued to determine the p24 antigen by the more reliable RIA.

From the 4th week p.i. all sheep were anti-p24 positive, the antibody titre being 1 : 400. From the 8th week p.i. the titre rose up to 1 : 1, 200, and at the 15th week p.i. the titre reached 1 : 2,400 to 1 : 3,200. In the non-treated animals the titre steadily increased to 1 : 6,000 to 1 : 10,000, during the 21st—25th weeks p.i. (Fig. 1). At that time we also determined the anti-gp51 titre. In the nontreated animals the anti-gp51 titre was 1 : 50,000, while in the treated animals the titre was only 2-fold lower (1 : 25,000). A similar difference between treated and non-treated animals was found with regard to the anti-p24 titre.

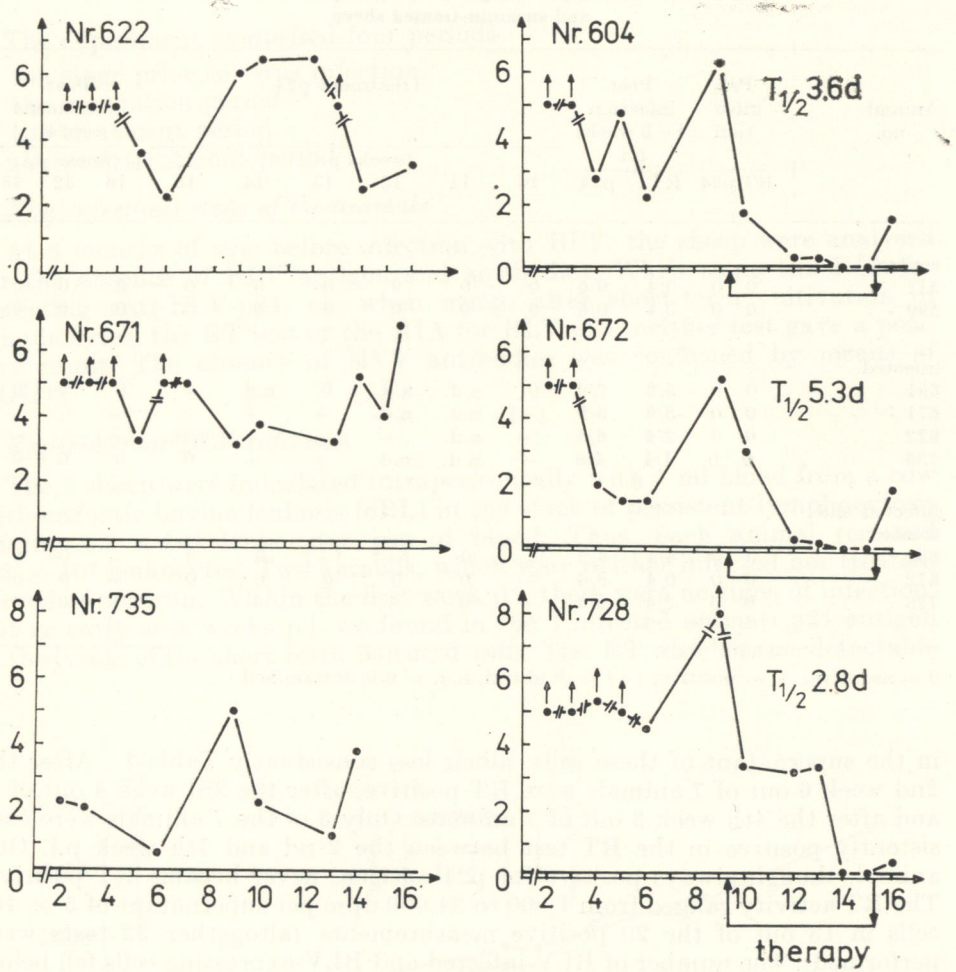

Fig 2.

Expression of p24 antigen in lymphocyte of BLV-infected sheep with and without suramin treatment

nos. 622, 671, 735: non-treated control sheep

nos. 604, 672, 728: sheep subjected to suramin treatment from the 10th till the 15th week p.i. The small arrows starting from some blacks dots indicate p24 values which were significantly higher than 5 ng/10^8 cells. The background level of p24 was 0.2 ng (horizontal line just above the abscissa). The treatment period is indicated by large arrows

Anscissae: weeks post-infection (onset and termination of therapy marked by large arrows); ordinates: log p24 per 10^8 cells.

Suramin therapy

After BLV infection had become apparent in the 7 sheep, 3 animals were used for suramin treatment, and 4 served as a control for following the infectious process. Suramin was administered intravenously once a week at a dose of 20 mg/kg for a period of 6 weeks. Four days after each injection blood samples were collected for p24 and anti-p24 detection. Suramin treatment led to a decrease in p24 levels. Table 2 gives a qualitative overview on the detection of p24 before, during and after suramin treatment. The course of the p24 levels during suramin treatment is presented in Fig. 2. The T1/2 values were 2.8, 3.6, 5.3 d. The p24 levels became undetectable within five weeks of treatment. This points to a beneficial effect of suramin therapy. Three out of the four infected non-treated control animals remained p24-positive throughout the whole period of treatment.

Post therapy period

During the post treatment period again all animals analysed for MVV infection were found to be negative. We also followed the anti-p24 titre and found that in the 7 infected animals, irrespective of whether or not they had been subjected to suramin treatment, the antibody titre was constantly high. The treated animals became again p24 antigen-positive by 1 week after stopping treatment (16th week). At the 32nd and 36th weeks these animals were again p24-negative. Yet, they turned out to be infectious: transmission of 5—10 ml blood to recipient sheep resulted in infection of the recipients. The non-treated control animals, irrespective of whether or not they were p24 antigen-positive, also proved to be infectious as monitored by this transmission experiment. Only the non-infected control animals remained non-infectious throughout.

Haematology

We did not see changes in the leukocyte numbers during the experiment, the cell numbers ranging from 4.5 to 10 $\times$ 10^3 per µl blood. The 7 BLV-infected individuals showed a weak increase of the leukocyte counts up to 7—10 $\times$ 10^3 per /µl blood during the first 3 weeks p.i. Cell counts returned to the original values thereafter, regardless of treatment or non-treatment. The lymphocyte percentage was about 70 % in the non-infected control animals and 74 % in the infected animals.

Discussion

Mammerickx *et al.* (1987) estimated that for sheep the minimal infectious dose of lymphocytes from cattle with BLV was about 1,000 cells (corresponding to about 300 infected cells). We decided to inoculate a high dose (4 $\times$ 10^7 leukocytes about 10^7 infected cells) in order to achieve infection within a short period of time.

Lymphocytes must be cultured for a short period of time to allow detection of antigen and RT. If one assumes that one BLV virion contains

3,000 p24 molecules (A. Burny, personal communication), 0.2 ng p24, the amount which could still be reliably detected by RIA, refers to 6.23×10^8 p24 molecules or 2×10^5 BLV particles per 10^7 cells. Thus, detection of BLV p24 antigen is by one order of magnitude more sensitive than detection of RT (Rössler *et al.*, 1980). The detection method of p24 is so sensitive that one can still find one BLV-infected cell among 50 non-infected lymphocytes (2 %).

As early as 1979 De Clercq showed that suramin, a polyanionic compound used for the treatment of sleeping sickness, is efficacious in inhibiting the RT of a number of retroviruses. The ID_{50} of suramin for AMV RT is about 0.1 μM (De Clercq, 1979). The RT of BLV is blocked at an ID_{50} of about 2.8 μmol/l suramin (Reimer *et al.*, 1989), 2.2 μmol/l 3′-FTTP (Matthes, personal communication), 0.17 μmol/l 3′-N_3TTP and 8.0 μmol/l HPA-23 (Reimer *et al.*, 1989). BLV RT is less sensitive to the above mentioned RT inhibitors than is HIV RT (Matthes *et al.*, 1987). In our experiment suramin was administered following the same regimen as used in the treatment of men suffering from protozoal infections, that is at 20 mg/kg/week intravenously for 6 weeks. Under these conditions we observed a suppression of p24 positive cells in the peripheral blood.

Our tests do not permit the detection of those cells that are infected and do not express the viral proteins. Suramin only inhibits infection of new cells by the actively virus producing cells. So, only after a certain period of time infected cells begin to produce virus, what makes them on the one hand detectable by our tests and on the other hand susceptible to suramin, which then blocks spread of the infection to new cells. However, infected cells which do not express antigen, survive and can express antigen after termination of therapy. The animals thus remain infectious, as was shown by the transmission experiments.

Our data could be interpreted as an indication for a positive effect of suramin, which together with the immunological response led to a drastic decrease of BLV-infected cells. The BLV-infected sheep may indeed serve as an animal model for the exploration of other retrovirus inhibitors or other therapeutic regimens. They may also serve as a model for the study of the pathogenesis of retroviral disease *in vivo*.

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