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A RADIOIMMUNOASSAY DETECTING THE BOVINE LEUKAEMIA VIRUS TRANSMEMBRANE PROTEIN GP30 AND ANTI-GP30 ANTIBODIES IN THE SERUM OF CATTLE

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Summary. — By means of SDS PAGE we isolated from virus-infected foetal lamb kidney (FLK) cells a relatively homogenous envelope transmembrane protein gp30 of bovine leukaemia virus (BLV). As shown by a partial sequence analysis of the N-terminus of this protein, our gp30 preparation contained only traces (less than 5 %) of p24 *gag* protein: Rabbit anti-gp30 serum did not cross react with the BLV proteins gp51, p12, $p15_1$, $p15_2$, and p10 but reacted weakly with the p24 polypeptide. ^{125}I-labelled gp30 (chloramine-T) was precipitated with the serum of BLV-infected cattle. Nonlabelled preparation of gp30 competitively inhibited the reaction of ^{125}I-labelled gp30 with the natural antibodies. We investigated 193 cattle sera by liquid phase radioimmunoassays (RIA) using ^{125}I-gp30, gp51 and p24 antigens. Sixteen noninfected cattle sera were negative in all tests. The 177 serum samples of BLV-infected animals were examined to the diagnostic value of the three tests. Of these, 175 were positive in gp51 RIA, 172 in p24 RIA and 164 in gp30 RIA. In all three tests, 159 sera were positive while 18 sera, mostly coming from animals with normal leukocyte counts, were positive only either with gp51 or p24, or were double positive with either gp51/p24 or gp51/gp30. We conclude that the gp51 RIA is superior to both the gp30 and the p24 RIA and that the gp30 RIA will be useful for investigating the role of gp30 in virus pathogenicity.

Key words: *bovine leukaemia virus*; *gp30*; *radioimmunoassay*

Introduction

Bovine leukaemia virus (BLV) is the causative agent of the enzootic bovine leukosis (EBL) (Burny *et al.*, 1980); it is a type-C retrovirus and a member of a distinct group of oncoviruses, which also includes HTLV-I and HTLV-II

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(Oroszlan *et al.*, 1982; Sagata *et al.*, 1983; Sagata *et al.*, 1985). The genome of BLV contains the coding regions *gag*, *pol*, *env*, and x (the latter having three open reading frames) (Gupta and Ferrer, 1980; Mamoun *et al.*, 1983; Sagata *et al.*, 1985). The *env* gene codes for the outer envelope protein gp51 and the transmembrane protein gp30 (Rice *et al.*, 1984). They originate from a common precursor protein with a signal peptide, $gPr^{env}72$ (Mamoun *et al.*, 1983; Ghysdael *et al.*, 1984; Rice *et al.*, 1984). The mature envelope protein gp51 consists of 268 amino acids and has 8 potential glycosylation sites, while the transmembrane protein gp30 consists of 214 amino acids with 2 potential glycosylation sites (Rice *et al.*, 1984). Schultz *et al.* (1984) have determined the sequence of 38 amino acids of the N-terminus and of 2 amino acids of the C-terminus of gp51 as well as the sequence of 12 N-terminal amino acids of gp30. From the DNA sequence coding for the transmembrane protein one can deduce several hydrophobic polypeptide regions (Rice *et al.*, 1984), the C-terminus of which is probably responsible for the anchorage within the envelope. An analogous situation was found in other retroviruses (Callis and Ritzi, 1980; Oroszlan and Nowinski, 1980). Within the envelope of intact virions gp51 and gp30 form homologous and heterologous dimers (Dietzschold *et al.*, 1978; Uckert *et al.*, 1982; Uckert *et al.*, 1984). Deshayes *et al.* (1977) and Deshayes *et al.* (1980) demonstrated the presence of humoral antibodies in BLV-infected cattle directed against the structural proteins $gp51^{env}$, $gp30^{env}$, $p24^{gag}$. The antigenic character of gp51 has been studied by means of monoclonal antibodies. Eight independent determinants were identified, three of which gave rise to neutralizing and cytolytic antibodies (Bruck *et al.*, 1982*a*; Bruck *et al.*, 1982*b*). Further on, gp51 agglutinates mouse erythrocytes (Sentsui, 1982). There are only speculations on the biological function of gp30. The transmembrane protein p15E of the feline leukaemia virus (FeLV) confers immunosuppressive effects on the host (Satake *et al.*, 1981), one might in analogy suppose that the immunosuppressive character of BLV is due to its gp30 (Denner *et al.*, 1986).

For pathogenicity studies and investigation of the role of gp30 in inducing antibodies or cell fusion or to assess the synthesis in microbial cells in gene technology, there is an urgent need of a sensitive test for the detection of gp30 protein and its antibodies. We describe here methods for obtaining gp30 from cultures of BLV-infected FLK cells, the construction of a liquid phase RIA, and obtaining monospecific antibodies against gp30. This RIA permits the detection of antibodies in the serum of BLV-infected cattle as well as in bacterial cultures expressing gp30 derivatives.

Materials and Methods

Cell culture and medium. Cells of the permanently BLV-infected FLK cell line (Van der Maaten and Miller, 1976) were cultured in Eagle's Minimal Essential Medium containing 10 % foetal calf serum, 200 IU penicillin per ml, 200 µg streptomycin per ml, and 25 IU nystatin per ml. The conditions of cell culture were as described by Scholz *et al.* (1984).

Sera. In order to check the diagnostic value of the gp30 RIA we investigated 193 serum samples; 16 animals of the Friedrich-Löffler-Institut, Insel Riems, which were negative in all tests for years, served as control group (Table 1). Another 152 animals (sera of groups 2, 3, and 4 of Table

Table 1. Detection of antibodies against BLV proteins gp51, p24, and gp30 in cattle

	group 1* (controls)		group 2		group 3 (persistent		group 4 lymphocytosis)		group 5 (animals with tumours)	
Leukocytes ($10^3/\mu l$ blood)	6.5		4.0—10.0		10.0—20.0		20.0		—	
No. of individuals	16		60		60		32		25	
Positive in gp51 RIA (%)	0	0	58	97	60	100	32	100	25	100
Positive in p24 RIA (%)	0	0	54	90	60	100	32	100	25	100
Positive in gp30 RIA (%)	0	0	52	87	55	92	32	100	25	100

* For classification of the animals into 5 groups see Materials and Methods

1) were from a milk giving herd consisting of 550 individuals followed up for more than 5 years by means of a commercially available AGID assay kit produced and distributed by Institut für Virologie, Humboldt-Universität Berlin. The 18 individuals listed in Table 2 belong to those of groups 2 and 3 from Table 1. The 25 individuals of group 5 of Table 1 are from different herds, they represent newborn calves and adult cattle both with clinically manifested tumours. All 177 sera are from naturally infected animals.

Isolation of BLV and purification of gp30. Cell culture supernatant (50 l) was collected for isolation of BLV according to Scholz *et al.* (1986). Virus particles were lysed and subjected to a preparative SDS PAGE in a 7.5—15% polyacrylamide gradient as described by Laemmli

Table 2. Detection of antibodies against BLV proteins gp51, p24, and gp30 in selected cattle*

Animal No.	Leukocytes ($\times 10^3$ µl)	Lympho-cytes ($\times 10^3$ µl)	AGID**	gp51 RIA	p24 RIA	gp30 RIA
1	6.2	4.2	—	+	—	—
2	5.6	3.2	—	—	+	—
3	5.9	3.4	—	—	+	—
4	5.2	3.4	—	+	—	+
5	7.7	5.3	—	+	—	+
6	6.8	3.9	—	+	—	+
7	5.7	4.6	+	+	—	+
8	6.1	3.2	(+)	+	—	+
9	7.6	3.2	—	+	+	—
10	6.3	4.0	—	+	+	—
11	4.6	2.8	+	+	+	—
12	5.7	3.6	+	+	+	—
13	5.4	3.6	(+)	+	+	—
14	10.1	7.5	—	+	+	—
15	10.3	5.1	—	+	+	—
16	10.2	3.8	—	+	+	—
17	12.5	10.0	(+)	+	+	—
18	15.0	10.0	+	+	+	—

* from groups 2 and 3 given in Table 1
** agar gel immunodiffusion test

(1970). After staining with Coomassie brillant blue G250, a protein band from the 30 kD region was eluted in phosphate buffer containing 0.1% SDS as described by Deshayes *et al.* (1977) and further concentrated through an Amicon PM10 filter.

Amino acid sequence analysis. Protein extracts in special degradation tubes (500 µl) were dried *in vacuo*. Sequential degradation was performed manually using the microsequence double coupling method of Chang *et al.* (1978) with dimethylamino azobenzene isothiocyanate (DABITC) (phenyl isothiocyanate/PITC). Residues of Coomassie blue did not interfere with thin layer chromatography identification of the dimethylamino azobenzene thiohydantoin amino acid. All operations concerning the removal of reagents by evaporation and drying were carried out *in vacuo* with a gentle stream of nitrogen using a double inlet valve.

Anti-gp30 antibodies. In order to obtain specific anti-gp30 antibodies, 100 µg SDS PAGE purified gp30 in 500 µl phosphate buffered saline (PBS) were mixed with an equal amount of complete Freund's adjuvant and injected into the footpads of a rabbit. Further injections contained 50 µg gp30 and incomplete adjuvant; one week after the last injection the animal was bled. The serum was incubated for 10 hr at +4 °C, centrifuged at 3,500 rev/min for 20 min and checked with an ELISA as described by Scholz *et al.* (1986).

Solid phase radioimmunoassay detection of specific antibodies. Amounts of 1 µg of isolated structural BLV proteins were fixed on cyanuric chloride activated paper (Hunger *et al.*, 1986), strips of which were incubated for 30 min at +25 °C in PBS containing 0.1% Tween 80 and 1% gelatine to block nonspecific binding sites. Specific antisera obtained as described above were added at 1 : 50 dilutions in PBS-0.1% Tween 80, and the strips were further incubated for 16 hr at +25 °C. Following extensive washings with PBS-0.1 %Tween 80 nonspecific activity was again blocked with PBS-0.1% Tween 80—1% gelatine for 30 min at +25 °C. The strips were then incubated with ^{125}I-labelled protein A from *Staphylococcus aureus* (1×10^6 cpm/ for 30 min, washed repeatedly, dried and exposed for autoradiography to ORWO XR1 film/VEB Filmfabrik Wolfen, D.D.R.). Control sera from rabbits were used in an identical manner.

Liquid phase radioimmunoassay for detecting anti-gp30 antibodies. Following SDS PAGE isolation gp30 was labelled with ^{125}I according to Greenwood *et al.* (1963) and used as antigen in a liquid phase radioimmunoassay described by Portetelle *et al.* (1980) and by Mammerickx *et al.* (1980).

Immunoblotting of E. coli/pEX recombinants with anti-gp30 antibodies. E. coli/pEX recombinants expressing a β-galactosidase/gp30 derivative fusion protein (Ulrich *et al.*, manuscript in press) were used for preparing the lysates. The lysate of 10^7 cells in 10 µl vol was subjected to SDS PAGE and electrotransferred onto nitrocellulose (0.025 mol/l Tris-HCl; 0.15 mol/l glycine pH 8.3 + 20% methanol; 4 hr, 400 mA). The filters were then blocked in PBS-0.1% Tween 80, pH 7.2, for 30 min. A 1 : 50 dilution of the specific anti-gp30 antiserum in PBS-0.1% Tween was used for incubation of the filters for 16 hr at room temperature, which then were subjected to extensive washings followed by an incubation with peroxidase-labelled anti-rabbit immunoglobulin (gift of VEB Kombinat Veterinär-Impfstoffe Dessau) at 1 : 500 dilution. Following careful washings in PBS-0.1% Tween, the reaction was visualized by adding a 2-bromo-1-naphthol solution (23 ml PBS, 6 mg substrate in 2 ml methanol, 10 µl 30% H_2O_2). After 30 min the filter was washed several times with bidistilled water and dried.

Results

Purification and identification of gp30

BLV purified and lysed as described above (40 mg) was subjected to SDS PAGE. The 30 kD protein band was eluted and reelectrophoresed until appeared homogenous (Fig. 1). On the basis of Coomassie staining the yield of gp30 was calculated to about 1 mg. Of this protein 150 µg was used to determine the first 6 amino acids of the N-terminus and by showing that these were NH_2-Ser-Pro-Val-Ala-Ala-Leu we were able to demonstrate that this protein was identical to gp30 (Schulz *et al.*, 1984) and that the impurities did not exceed 5 %.

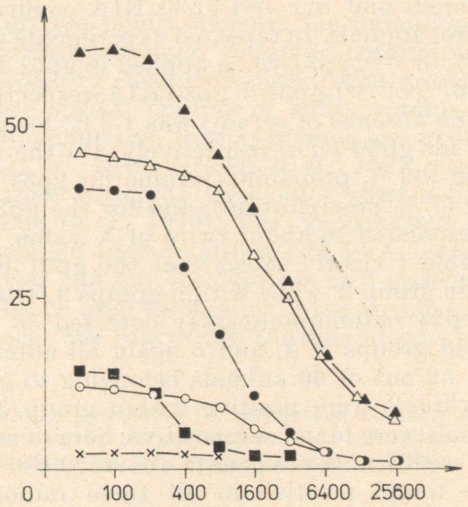

Fig. 2.
Detection by liquid phase RIA of anti-gp30 antibodies in the sera of BLV infected cattle
The test tubes contained sera diluted from 1 : 100 to 1 : 25,600 and about 15,000 cpm of ^{125}I-gp30 in a total volume of 400 µl RIA buffer. △ and ○ = sera from animals with persistent lymphocytosis; ▲, ●, and ■ = sera from animals with tumours; × = negative control serum.

Rabbit anti-gp30 antibodies

To prepare the rabbit antiserum (titre 1 : 500) we used 300 µg gp30. The serum reacted in solid phase RIA using a cyanuric chloride activated (CCA) paper (Hunger *et al.*, 1986) with a galactosidase-gp30 fusion protein from genetically engineered *E. coli* (Ulrich *et al.*, manuscript in press), with the isolated gp 30, and with BLV. The serum was also shown to react with the isolated gp30 and with BLV in a solid phase RIA (Uckert *et al.*, 1986). It neutralized the infectivity of pseudotypes containing VSV genome and BLV envelope (J. Závada, pers. communication).

Detection of specific antibodies in cattle by gp30 radioimmunoassay

The iodinated purified gp30 showed one single band located within the 30 kD range (Fig. 1). We checked this radioactive gp30 as to whether it was precipitated with sera of BLV-infected cattle using liquid phase RIA according to Portetelle *et al.* (1980). Fig. 2 shows the titration curves of 5 randomly selected sera which were previously found positive to gp51 and p24 in independent assays. Of the ^{125}I-gp30 20—70 % was precipitated. The serum of one animal which appeared to be negative for both anti-gp51 and anti-p24 precipitated as little as 4.5 % of the ^{125}I-gp30, which was regarded as negative. Addition of cold gp30 diminished the amount of precipitated ^{125}I-gp30 (data not shown).

In order to determine the sensitivity of the liquid phase RIA described above we investigated 193 animals with respect to anti-gp30, anti-gp51 and anti-p24 employing the same type of RIA. On the basis of haematological, clinical and serological data the animals were divided into 5 groups (Table 1). Control sera (16 samples) negative in anti-gp51 and anti-p24 RIA were also

included, and our ^{125}I-gp30 RIA confirmed the BLV-free status of these control animals. In repeated experiments these 16 sera precipitated in average 5.5 % of ^{125}I-gp51 when applied in gp51 RIA, 3.5 % of ^{125}I-p24 in p24 RIA, and 5 % of ^{125}I-gp30 in gp30 RIA, respectively. The background precipitation (in the absence of serum) was 1.3 % for gp51 RIA, 0.5 % for p24 RIA, and 1 % for gp30 RIA, respectively. On the basis of these data we calculated a cutoff (99 % probability) value for gp51 RIA (12 % precipitation), for p24 RIA (7 % precipitation), and for the gp30 RIA (10 % precipitation) which corresponded to about twice of X value.

Table 1 clearly shows that the gp51 RIA detected 58 out of 60 animals within group 2, while within groups 3, 4, and 5 all animals reacted positively. The p24 radioimmunoassay detected 55 out of 60 animals within group 2, and in groups 3, 4, and 5 again all animals reacted positively. Using gp30 RIA 52 out of 60 animals belonging to group 2 were positive, 55 out of 60 individuals were positive within group 3, while within groups 4 and 5 all animals were found seropositive. Sera of cattle with persistent lymphocytosis, sera with leukocyte counts above 20,000 per µl and sera of tumour animals were found positive in all three radioimmunoassays (gp51, gp30, p24). Table 2 shows the data of 18 individuals whose sera differed in the results of the three RIAs. With 16 sera anti-gp51 antibodies were detectable, 5 of which had also gp30 antibodies. 10 of 18 sera in question had p24 antibodies. Two sera reacted exclusively in the p24 RIA, one serum only in the gp51 RIA. Antibodies against gp30 were detected only in connection with anti-gp51 antibodies. The data clearly show the reliable differentiation of the antibody reaction against gp30 and p24 when using sera of infected cattle. Those 5 sera which react both in the gp51 RIA and gp30 RIA but not in the p24 RIA testify the specificity of our gp30 RIA based on the high degree of purity of the ^{125}I-gp30 antigen.

Discussion

Sera of BLV-infected cattle precipitated the transmembrane protein gp30 prepared and iodinated as described. The reaction of ^{125}I-gp30 could be blocked by cold gp30. The assay was positive in BLV infected haematologically and clinically normal animals as well as in animals with persistent lymphocytosis; in contrast Deshayes *et al.* (1980) detected anti-gp30 antibodies by means of a modified radioimmunoassay (RIPA) only as late as in the tumour stage of EBL. Therefore, our test broadens the repertoire of diagnostic tools. Antibodies against gp30 seem to appear early after infection. However, the frequency of their appearance seems to be somewhat lower if compared with that of antibodies against gp51 and p24. This might be due to the SDS treatment and/or the iodination procedure which might decrease the immunogenic potential of the gp30 antigen by destroying some antigenic determinants. It is also possible that the hydrophobic character of gp30 (Rice *et al.*, 1984), renders the protein less immunogenic. Callis and Ritzi (1980) reported similar data when comparing the reaction of transmembrane protein with other proteins of mouse breast tumour virus. It is also possible

that the SDS treatment and/or iodination might decrease the immunogenic potential of the gp30 antigen by destroying some antigenic determinants. The appearance of anti-gp30 antibodies in close connection with anti-gp51 antibodies could be explained by the special binding of both proteins and by the anchorage of gp30 within the membrane, so that gp30 is less exposed as compared to gp51 (Dietzschold *et al.*, 1978; Uckert *et al.*, 1982; Uckert *et al.*, 1984). Our results confirm that anti-gp51 antibodies most reliably indicate BLV infection in cattle (Bex *et al.*, 1979). The confirmation of a negative status of EBL, however, should include also a p24 RIA, as some animals, do react only with the latter. As our test detects anti-gp30 antibodies in an early stage of EBL, it can be used for studies of the pathogenesis of BLV infection especially as concerns the role of the transmembrane gp30 protein.

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Explanation to Figure (Plate XVI):

Fig. 1. Separation by SDS PAGE of BLV Proteins
40 mg BLV protein was subjected to 7.5–15.0 % SDS PAGE using 6 V cm^{-1} (B). Molecular weight markers: 14 kD -lactalbumin; 20 kD soybean trypsin inhibitor; 30 kD carbonic acid anhydratase; 43 kD ovalbumin; 48 kD bovine serum albumin; 92 kD phosphorylase (A). Electrophoresis of isolated gp30 (10 µg after Coomasie blue staining and concentration according to Materials and Methods) is given in C. ^{125}I-labelled gp30 (15,000 cpm) was run in D.