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Molecular Dissection of the Bovine Leukemia Virus Envelope Glycoprotein (BLV gp51) by a Monoclonal Antibody Study*

C. Bruck, D. Portetelle, J. Zavada, and A. Burny

A. Introduction

Neoplasms of the lymphatic tissue in the bovine species can be classified into two major types according to clinical and epidemiological data:
1. The juvenile form of bovine leukosis, with three different clinical forms: the multicentric, thymic, and cutaneous forms. The juvenile form is rare and shows a random geographical distribution. It has also been called sporadic bovine leukosis.
2. The adult form of bovine leukosis leads to more diversely located lymphosarcomas, mostly of the B-lymphocyte lineage and/or to B-cell leukemias. The adult form of bovine leukosis is more common, but restricted to geographically limited regions. Although it affects only a small percentage of the cattle population, it behaves as a typical herd disease. Its distribution pattern is typically one of transmissible diseases and has allowed the classification of the adult form of lymphosarcomas as "enzootic bovine leukosis" [1].

In 1969 Miller et al. identified viral particles in short-term cultures of leukocytes of animals in persistent lymphocytosis [2]. Transmission experiments [3] and seroepidemiological studies [4] finally established that this virus is an etiological agent of enzootic bovine leukosis. This virus, called bovine leukemia virus (BLV), can be experimentally transmitted to sheep, goats, primates, rabbits, and other mammals by intravenous, intradermic, or oral administration of infected lymphocytes. Sheep are highly susceptible to leukosis induction by BLV, goats rarely develop BLV lymphosarcomas, and other animals, although persistently infected by BLV, seem to be resistant to the oncogenic properties of this virus [1]. Only one case of tumor induction in goats by BLV has been reported so far [5]. In natural conditions, BLV is transmitted between cattle by "close contact". The exact mode of transmission in the field condition is still unclear: transmission by infected animals, secretions and milk, blood-sucking insects, and nonsterile veterinary instruments have been reported.

Molecular analysis of BLV has made possible its classification as a retrovirus (60–70S genomic RNA, reverse transcriptase) [6]. Hybridization experiments with cDNA complementary to genomic viral RNA have established the following points:
1. BLV is an entirely exogenous virus: integrated proviral BLV DNA is only detectable in infected B-lymphocytes [7].
2. No proviral DNA can be found in sporadic leukosis tumors [8]; enzootic leukosis tumors always contain one or several copies of proviral DNA. The integration site of the BLV genome is variable from case to case [9].
3. The three viral genes necessary for the virus life cycle have been identified on the BLV genome (*gag, pol, env*), showing that BLV is a nondefective leukemia virus [10].

Immunochemical studies have led to the identification of the viral glycoproteins and proteins [1]: gp51 and gp30 are envelope

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antigens; p24 is the major core protein; reverse transcriptase is associated with the genomic RNA; p15, p12, and p10 are internal proteins with so far undefined location and function.

BLV is genetically and antigenically unrelated to other animal retroviruses and was considered as a group of its own until the discovery of a human T-cell lymphoma retrovirus HTLV in 1981 [11]. The major internal core protein of HTLV p24 shares amino acid sequence homology with BLV p24, and HTLV and BLV p12 display common antigenic determinants [12]. Like HTLV, BLV is a weakly oncogenic virus: there is a long latency between the moment of BLV infection and the onset of the tumor phase, and only a small percentage of the infected animals die with leukemia: in Japan 30%–50% of clinically normal cattle in an endemic area became infected with BLV, while the incidence of lymphosarcoma or leukemia in this area was less than 0.1% [13].

In about 30% of infected cattle, BLV infection leads to a nonmalignant hematological disorder characterized by an elevated but stable lymphocyte count, called persistent lymphocytosis (PL). As shown by molecular hybridization, the expanded lymphocyte population in the PL phase is of polyclonal origin: the proviral BLV is integrated at many different sites, whereas the tumor phase is monoclonal [14]. These observations, together with the weak oncogenic activity of BLV, suggest that a condition superimposed on BLV integration is necessary to induce neoplastic transformation of lymphoid cells.

Natural or experimental infection of cattle with BLV induces a vigorous antibody response, which is often the only constant feature of BLV infection during the latency period of the disease. Antibodies are directed mainly against the envelope glycoprotein gp51 and the internal core protein p24 [1]. Antibodies to gp51 are consistently formed at a higher titer than antibodies to p24 and can be detected earlier after experimental infection [15]. In spite of their high titer, antiviral antibodies do not seem to be protective against the onset of the leukemic phase: although subject to major fluctuations, the antibody titer rises constantly during the progression of the disease and reaches maximal level at the death of the animal in the tumor phase [16].

May be as a consequence of antigenic modulation, BLV-infected lymphocytes do not express viral antigens in vivo [17]. This could explain why antiviral antibodies produced in response to BLV infection are unable to inhibit the outgrowth of leukemic clones. However, passive immunization of calves with colostral antibodies is protective against primary BLV infection [18], suggesting that efficient vaccination against primary BLV infection should be possible.

Previous studies have suggested that the natural anti-gp51 antibody response is focused on a single antigenic region of gp51. Exoglycosidase treatment of gp51 abolishes the reactivity of these antibodies against the antigen, suggesting that the structure of the relevant antigenic region is directly or indirectly determined by the carbohydrate moiety of the molecule [19]. These natural anti-gp51 antibodies produced by infected cattle display several antiviral activities: they neutralize virus infectivity [20] and syncytia-inducing activity [21] and exhibit a strong cytolytic effect on BLV-producing cells in the presence of rabbit complement [16]. In order to characterize the regions of gp51 which are indispensable for an efficient BLV vaccine, monoclonal anti-gp51 antibodies were produced.

B. Results

Fifteen monoclonal anti-gp51 antibody-secreting hybridoma cell lines were obtained by fusing spleen cells of a Balb/c mouse immunized with partially purified gp51 with a subclone of the SP_2 myeloma cell line. These antibodies were classified into high- and low-avidity antibodies according to the slope and plateau values of their titration curves obtained by ELISA titers on polyethylene immobilized gp51 (see Table 1). Competition experiments between these different antibodies registered the fixation of radiolabeled antibodies in the presence of an excess of cold competition antibody. This test allowed the identification of eight nonoverlapping gp51 epitopes (A–H). Partially overlapping sites have been called B and B′, and D and D′ (see Table 1, Fig. 3).

Table 1. Pseudotypes and early polykaryocytosis: inhibition activities of 15 Anti BLV gp51 Monoclonal antibodies

Antibody number	Ig isotype	Binding activity on plastic-adsorbed gp51	Site specificity	Number of plaques in PI test	Number of Syncytia in EPI
GA1	IgG1	High	A	155	233
GA2	IgG1	High	B	102	268
GA3	IgG1	Low	H	50	1
GA4	IgG1	Low	F	39	151
GA5	IgG1	High	D	140	210
GA6	IgG2b	Low	E	102	258
GA7	IgG1	Low	E	120	210
GB8	IgG1	High	C	100	298
GB9	IgG1	High	A	121	225
GB10	IgG1	High	B′	114	210
GB11	IgG1	High	A	125	320
GA12	IgG1	Low	E	NT	NT
GA13	IgG1	Low	H	4	0
BC14	IgG2a	Low	G	33	125
GA15	ND	High	D′	127	265

In order to localize these antigenic sites on the gp51 molecule, we performed limited protease (urokinase) digestion of the antigen, followed by radioimmunoprecipitation (RIP) with monoclonal antibodies and SDS-PAGE analysis. The results show that the eight sites are distributed on two distinct fragments of gp51, sites A–D on fragment I, mol. wt. 35,000, sites E–H on fragment II, mol. wt. 15,000 (antibodies against sites E–H recognize each of the four bands of different molecular weight, suggesting that urokinase digestion of gp51 is incomplete) (see Table 1, Fig. 1).

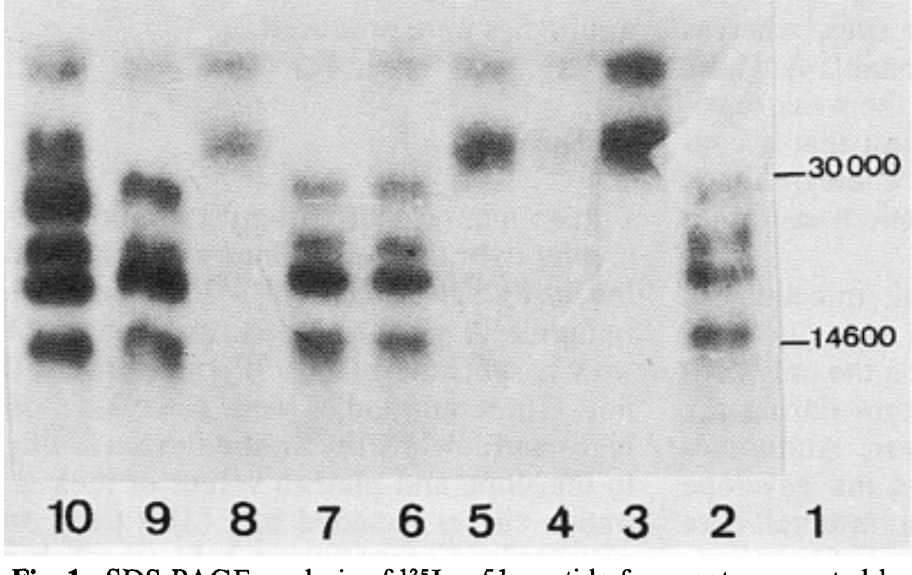

Fig. 1. SDS-PAGE analysis of ^{125}I gp51 peptide fragments generated by limited urokinase digestion and precipitated by the individual monoclonal antibodies. *Lane 1,* molecular weight markers; *lane 2,* peptide fragments precipitated by BC14 (site G); *lane 3,* peptide fragments precipitated by antibodies GA2 and GB10 (site BB′); *lane 4,* peptide by antibodies GB8 (site C); *lane 5,* peptide fragments precipitated by antibodies GA1, GB9, and GB11 (site A); *lane 6,* peptide fragments precipitated by antibodies 6A3 and GB13 (site H); *lane 7,* peptide fragments precipitated by antibodies GA4 (site F); *lane 8,* peptide fragments precipitated by antibodies GA5 and GB15 (site DD′); *lane 9,* peptide fragments precipitated by antibodies GA6, GA7, and GA12 (site E); *lane 10,* unfractionated gp51 peptide fragments

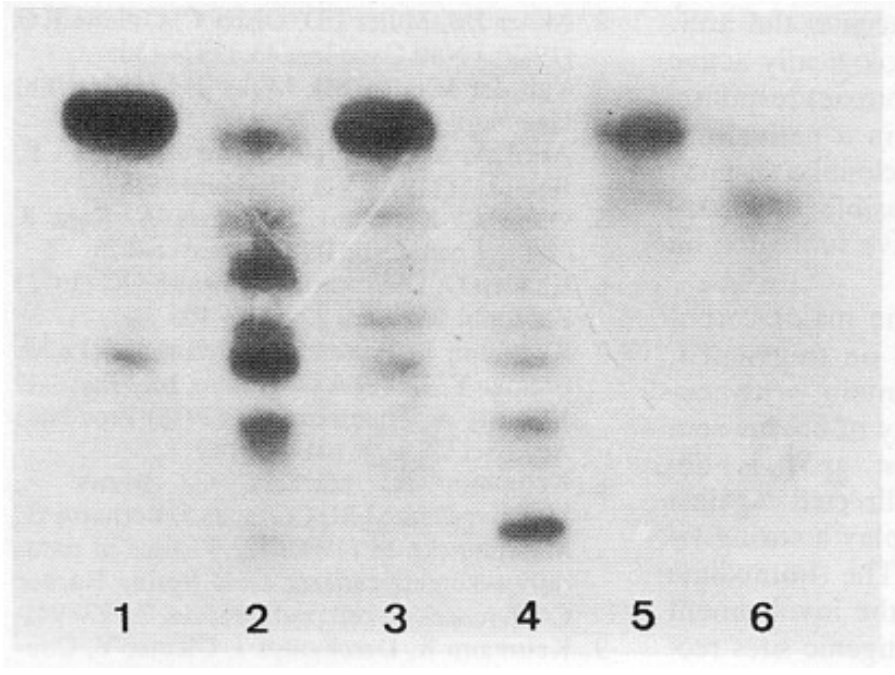

Fig. 2. SDS-PAGE analysis of gp51 peptide fragments generated by limited urokinase digestion. *Lane 1,* undigested ^{125}I-labeled gp51; *lane 2,* UK digested ^{125}I-labeled gp51; *lane 3,* undigested lysyl-specific ^{3}H-labeled gp51; *lane 4,* UK digested lysyl-specific ^{3}H-labeled gp51; *lane 5,* undigested, galactose-specific ^{3}H-labeled gp51; *lane 6,* UK digested, galactose-specific ^{3}H-labeled gp51

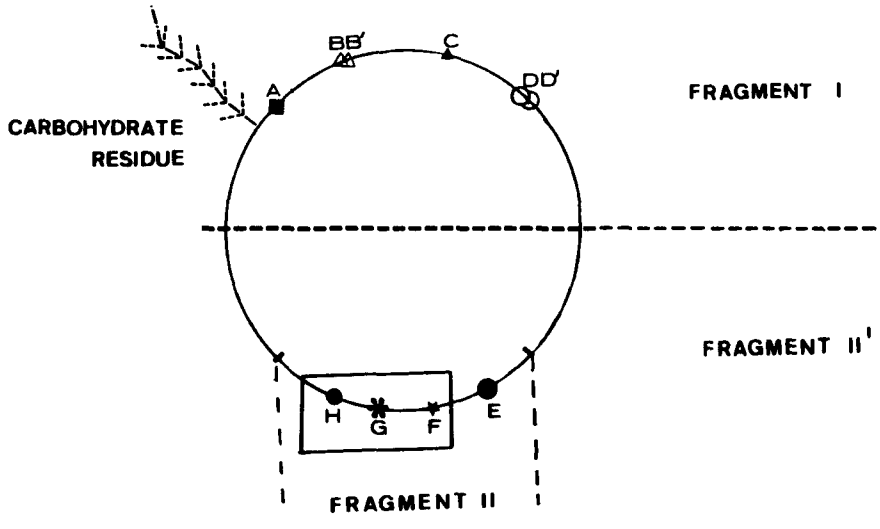

Fig. 3. Model for the location of the epitopes recognized by mouse monoclonal antibodies on the gp51 molecule

Galactose-specific labeling of gp51 followed by urokinase digestion and SDS-PAGE analysis revealed that fragment I of mol. wt. 35,000 contains the major carbohydrate residues of the molecule (Fig. 2). This enabled us to establish a model for the location of the different epitopes at the surface of the gp51 molecule (Fig. 3). In order to identify the epitopes of gp51 involved in virus neutralization and cytotoxicity toward virus-producing cells, we tested the 15 antibodies for:

1. Pseudotype inhibition activity according to Zavada et al. [20].
2. Early polykaryocytosis inhibition activity according to Guillemain et al. [21].

Furthermore, the monoclonal Ab BC14, of Igb2a subclass, was tested for its complement-dependent cytotoxicity toward BLV-producing cells according to Portetelle et al. [16].

The results showed that three of the four epitopes located on the 15,000 mol. wt. nonglycosylated fragment are involved in virus neutralization, and that at least one of these epitopes is also a target for cytotoxic antibodies on virus-producing cells (see Table 1, Fig. 3). Antibodies against fragment I, which contains the major carbohydrate residues of the molecule, displayed none of the biological activities tested for.

C. Discussion and Conclusion

A detailed knowledge of the different antigenic regions of the gp51 molecule is necessary for the development of a BLV

vaccine. In an efficient vaccine, the antigenic regions eliciting a biologically active (virus-neutralizing and cytotoxic) response, must be fully represented in a native, undamaged form. Our monoclonal anti-gp51 antibodies have made possible the mapping of the biologically active subregion on the antigenic molecule.

The results show that the major carbohydrate chains are located on fragment I, which does not seem to contain virus-neutralizing sites. The reactivity of bovine sera (which is abolished when gp51 is deglycosylated) is mostly directed against fragment II, since they display a strong virus-neutralizing activity. The immediate conclusion would be that the involvement of carbohydrates in the antigenic sites recognized by sera of infected cattle is indirect rather than direct, and that removal of carbohydrate chains changes the three-dimensional structure of the molecule and can influence a distal antigenic site. However, the existence on fragment II of small carbohydrate core structures devoid of galactoside residues, which would account for the antigenic properties of gp51 in cattle, cannot be excluded yet.

Our results show that only a fragment of the gp51 molecule is involved in fixation to the cellular receptor, i.e., infectivity. The glycosylated fragment of 35,000 mol. wt. might play a role in providing proper exposure of the active site on the viral particle. BLV-neutralizing monoclonal antibodies can be used as probes for the detection of these important epitopes in the development of a BLV vaccine.

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