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Polyclonal Bovine Sera but Not Virus-Neutralizing Monoclonal Antibodies Block Bovine Leukemia Virus (BLV) gp51 Binding to Recombinant BLV Receptor BLVRcp1

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Bovine leukemia virus (BLV), a transactivating lymphotropic retrovirus, is the etiologic agent of enzootic lymphosarcoma or leukemia in cattle. Sera from BLV-infected animals possess high BLV-neutralizing antibody titres. The availability of the recombinant BLV receptor candidate, BLVRcp1, allowed us to determine a mechanism of virus neutralization by polyclonal sera and monoclonal antibodies (MAbs). Bovine sera from animals naturally infected with BLV blocked gp51 binding to recombinant BLVRcp1. In contrast, virus-neutralizing MAbs specific for gp51 F, G, and H epitopes did not prevent gp51-receptor attachment. Furthermore, gp51 neutralization epitopes F, G, and H were accessible to antibodies following gp51 attachment to BLVRcp1. This finding implies that virus neutralization by MAbs to defined BLV gp51 epitopes can occur subsequent to virus engagement of the receptor while polyclonal sera can specifically block virus attachment to the receptor. In conclusion, these data suggest that cell infection by BLV is a multistep process requiring receptor binding (inhibited by polyclonal sera) followed by a second, postbinding event(s) at the cell membrane (inhibited by anti-gp51 MAbs).

Bovine leukemia virus (BLV) is a lymphotropic retrovirus of cattle that is closely related to human T-cell lymphotropic viruses 1 and 2 and causes enzootic lymphosarcoma/leukemia in a small percentage (0.6 to 5%) of infected animals. Up to 30% of infected animals develop persistent lymphocytosis, a benign and permanent increase in circulating B lymphocytes; however, these animals remain asymptomatic (21, 36). Seroconversion and lifelong presence of antibodies are the only indications of infection in the vast majority of infected animals (9, 18). Antibody response to BLV infection can be detected as soon as 2 weeks after infection, and antibody titer increases can be detected with time (24). BLV structural proteins, predominantly p24 and gp51, are targets for antibody response, and detection of antibodies is routinely used for diagnosis of BLV infection (5, 34). Antibodies to the transactivating protein Rex can be detected intermittently, and their occurrence likely correlates with episodes of Rex release from dead (or killed) infected cells (35). Antibodies from infected animals are virus neutralizing in vesicular stomatitis virus-BLV pseudotype neutralization and syncytium inhibition assays (4, 41) and mediate lysis of infected cells by complement fixation (31). Humoral immunity likely plays an important role in protection from progression of BLV infection. Passive immunization with anti-BLV antibodies conferred protection from BLV infection (22). Resistance to BLV challenge after vaccination of sheep with gp51 antigen (27) and cattle with cells producing BLV *env*-encoded glycoproteins and p24 (2) correlated with the presence of virus-specific antibodies. Despite the fact that tumors develop in the presence of antibodies, selection pressure executed by antibodies can be a key host factor, inducing virus latency, absence of viremia, and a long incubation period prior to tumor occurrence.

Immunological analysis using monoclonal antibodies (MAbs) revealed two immunodominant B-cell epitope regions on the main structural protein, p24, of BLV (29). Similar analysis of envelope glycoprotein gp51 characterized eight distinct epitopes (11), and two additional epitopes were detected later (28). Linear epitopes A, B-B′, D, and E are not neutralizing and are localized near the carboxy-terminal end of gp51 (6, 14). Epitopes F, G, and H are conformational and virus neutralizing and localized at the amino-terminal end of gp51 (32, 33). Epitopes C, C1, and C2 are conformational, without virus-neutralizing activity (11, 28).

Recently, a cDNA encoding the putative BLV receptor, BLVRcp1, was isolated and characterized (7, 8). Transfection of the BLVRcp1 cDNA into nonpermissive NIH 3T3 cells conferred sensitivity to BLV infection, and infection of transfected cells was prevented by anti-gp51 neutralizing MAbs to epitopes F, G, and H. BLVRcp1 cDNA encodes a 94-kDa protein in MDBK cells, and its chromosome localization in several species was determined (30); however, the physiological function of this protein has not been determined. The availability of putative BLV receptor BLVRcp1 provided the opportunity to study the engagement of viral gp51 with its receptor and investigate the mechanism of virus neutralization by polyclonal sera and MAbs.

Recombinant BLVRcp1 preparation and characterization. To prepare recombinant receptor, the BLVRcp1 *Eco*RI-*Xba*I fragment (7) was inserted into the plasmid pMAL-c2 (New England BioLabs, Beverly, Mass.). Transformation of *Escherichia coli* DH5α resulted in expression of a fusion product containing maltose binding protein (MBP). Bacterial lysates of isopropylthiogalactoside-induced bacterial cells from the clone MBP-BLVRcp1, which is MBP containing BLVRcp1 cDNA,

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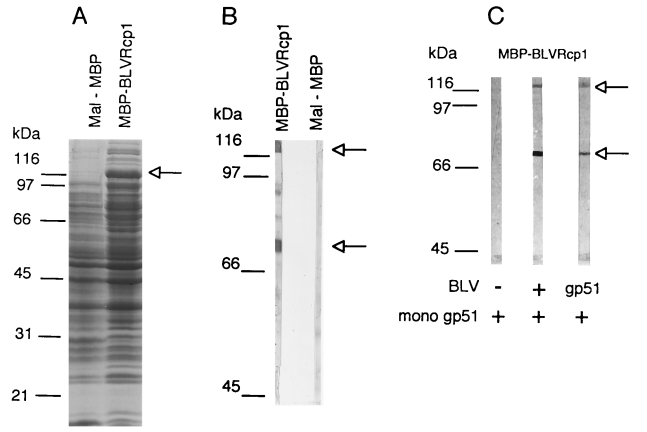

FIG. 1. Characterization of recombinant fusion product of BLVRcp1. (A) Bacterial proteins were separated by SDS-PAGE on a 10% gel and visualized by staining with Coomassie blue. MBP-BLVRcp1, bacterial lysate containing MBP-BLVRcp1 fusion protein; Mal-MBP, control lysate. The arrow indicates the overexpressed, 120-kDa MBP-BLVRcp1 recombinant protein. (B) Western blot of bacterial lysates detected with rabbit antiserum R70 raised against amino acids 25 to 36 of BLVRcp1. Arrows indicate specific binding to the full-length, 120-kDa MBP-BLVRcp1 and its 75-kDa degradation product. (C) Receptor binding assay on MBP-BLVRcp1 lysate detected by a pool of MAbs to gp51 epitopes A to H. BLV, tissue culture fluid from virus-producing FLK cell line containing disrupted BLV; gp51, BLV gp51 purified by affinity chromatography. Arrows indicate gp51 binding to the full-length, 120-kDa MBP-BLVRcp1 and its 75-kDa degradation product.

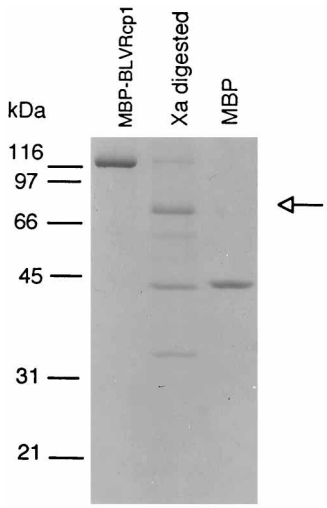

FIG. 2. Electrophoretic analysis of purified MBP-BLVRcp1 subjected to SDS-PAGE (10% polyacrylamide) and stained with Coomassie blue. MBP-BLVRcp1, full-length, purified fusion MBP-BLVRcp1 protein; Xa digested, MBP-BLVRcp1 protein after digestion with factor Xa. The arrow indicates recombinant BLVRcp1 protein separated from MBP.

and the mock-transfected clone Mal-MBP were analyzed for expression of recombinant protein. Comparison of electrophoretic profiles in Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels revealed the presence of a recombinant product with the predicted size 120 kDa in the lysate MBP-BLVRcp1 but not in the lysate Mal-MBP (Fig. 1A). To determine the antigenic authenticity of the recombinant MBP-BLVRcp1 fusion product, a rabbit antiserum, R70, raised against amino acids 25 to 36 of BLVRcp1, was used (7). In Western blot analysis, R70 diluted 20,000× followed by incubation with secondary alkaline phosphatase (AP) conjugate and chromogenic substrate nitroblue tetrazolium chloride (NBT)–5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) specifically recognized a 120-kDa fusion product and its 75-kDa degradation product in the MBP-BLVRcp1 lysate. Bacterial lysate Mal-MBP did not react with the peptide antiserum R70 (Fig. 1B). Preimmune rabbit serum R70 did not bind to MBP-BLVRcp1 (data not shown).

Furthermore, MBP-BLVRcp1 protein was tested in a receptor binding assay for binding of affinity-purified gp51 (25) or gp51 from FLK-VP-1 (ovine) (1), R(BLV) (rat), (3), BK(BLV) (bovine), or B/BLV (bat) cell line supernatants containing virions disrupted by 0.1% *N*-octylglucoside. Nitrocellulose membranes blotted with MBP-BLVRcp1 lysate were blocked with 5% nonfat milk in phosphate-buffered saline and incubated with purified gp51 or disrupted virions in TBS (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20) for 16 h at 4°C. Glycoprotein gp51 binding to the recombinant receptor was detected with a pool of MAbs to gp51 epitopes A to H (11, 28) followed by anti-mouse AP-labelled secondary antibody and chromogenic substrate NBT-BCIP. As shown in Fig. 1C, full-length, 120-kDa fusion recombinant MBP-BLVRcp1 as well as its 75-kDa degradation product bound gp51. Recombinant MBP-BLVRcp1 degradation was difficult to control, and various degradation patterns were observed with different lysate preparations. The bacterial lysates used for experiments in Fig. 1, 3, and 5 were different. Bacterial lysate with the least degradation was observed in Fig. 5, in which only full-length, 120-kDa MBP-BLVRcp1 bound gp51. Two additional degradation products, 25 and 75 kDa, bound gp51 in the lysates shown in Fig. 3 or Fig. 1, respectively. BLV gp51 from all sources tested bound recombinant fusion BLVRcp1. Binding of gp51 to nitrocellulose strips with Mal-MBP lysate was not detected (data not shown).

Next, we purified the recombinant fusion product from bacterial lysate by affinity chromatography on amylose resin columns (New England BioLabs). However, binding to the column was poor and several degradation products were copurified (data not shown). Therefore, nondegraded, full-length MBP-BLVRcp1 protein was recovered after excision and electroelution of the 120-kDa band from SDS-PAGE gels (19). Specific cleavage of the fusion protein with factor Xa resulted in the appearance of two expected major protein bands, 75 and 42 kDa, corresponding to BLVRcp1 protein and MBP, respectively (Fig. 2). However, digestion with factor Xa was incomplete, with a minor, 120-kDa-band residue and nonspecific cleavage resulted in production of several minor degradation bands of 50, 60, and 66 kDa, while the 35-kDa band corresponded to factor Xa. Because purified and digested BLVRcp1 yielded small amounts and degraded protein, MBP-BLVRcp1 bacterial lysate was used for BLVRcp1-gp51 blocking experiments.

Inhibition of BLVRcp1-gp51 binding by MAbs. Previously, the amino-terminal portion of BLVRcp1 protein, encoded by the *Sma*I-*Sac*I DNA fragment, was demonstrated to be crucial for gp51 binding and comprises the receptor binding site (7). However, the receptor binding site on gp51 has not been clearly identified. Therefore, we tested whether some of the previously characterized gp51 epitopes A to H might comprise the gp51 receptor binding domain (11, 28). Eleven individual gp51-specific MAbs or smaller pools of MAbs were tested to detect gp51 bound to BLVRcp1. Surprisingly, binding of gp51 to the 120-kDa, full-length MBP-BLVRcp1, as well as the 25-kDa degradation product was detected by pools of MAbs as well as all individual anti-gp51 MAbs (Fig. 3). Binding to the receptor was detected by antibodies only with addition of gp51,

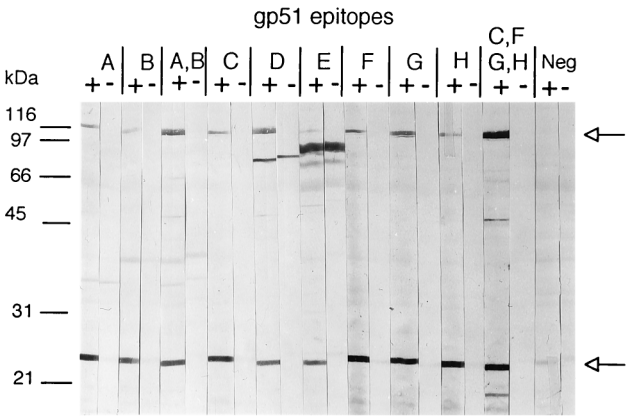

FIG. 3. Virus binding assay using MBP-BLVRcp1 lysate detected by individual anti-gp51 MAbs and pools of MAbs. Nitrocellulose strips were incubated in tissue culture fluid containing (+) or lacking (−) gp51 from disrupted BLV. Bound MAbs were visualized by reaction with AP-conjugated anti-mouse secondary antibody and NBT-BCIP chromogenic substrate. Individual letters A to H indicate gp51-epitope specific MAbs used for detection. A,B and C,FG,H are pools of MAbs. MAb BLVp24-X48 possessing BLV anti-p24 specificity was used as a negative control (Neg). Arrows indicate gp51 binding to the full-length, 120-kDa MBP-BLVRcp1 and a 25-kDa degradation product.

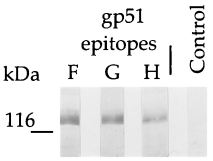

FIG. 4. Neutralization of gp51-BLVRcp1 binding by neutralizing MAbs. Medium containing gp51 was preincubated with individual MAbs prior to incubation with the BLVRcp1 blot. gp51-MAb immune complexes were detected by secondary AP-conjugated anti-mouse secondary antibody and chromogenic substrate. Letters F to H designate the epitope specificities of the gp51 neutralizing MAbs. MAb BLVp24-X48 was used as a control antibody.

confirming the specificity of the reactions. Protein bands detected on Western blots incubated without BLV represent nonspecific binding of relevant MAbs to bacterial lysate, particularly evident with MAb to epitope E (Fig. 3). Control MAb to BLV p24 (29) did not react with bound gp51. These data indicated that none of the gp51 epitopes A to H are directly involved in the receptor binding site and that virus-neutralizing epitopes F, G, and H were always exposed after gp51 receptor engagement. This finding was unexpected, because MAbs to these epitopes are virus neutralizing and inhibition of virus-receptor interaction was originally proposed as the mechanism of virus neutralization by MAbs (11). However, our data indicate that this explanation may not be correct. Furthermore, a pool of MAbs to conformational epitopes was superior for detecting BLVRcp1-bound gp51 (Fig. 3). The finding that virus-neutralizing MAbs can bind to receptor-bound gp51 is directly applicable in understanding the action of virus-neutralizing MAbs against gp51. Maintaining accessibility of neutralization epitopes F, G, and H after gp51 binding to the receptor indicates that neutralizing MAbs elicit their function following virus attachment to the receptor.

To dissect the mechanism(s) of virus neutralization by MAbs, MAbs recognizing gp51 F, G, and H epitopes were tested for blocking gp51 binding to BLVRcp1 in the receptor binding assay. Medium containing gp51 was preincubated overnight with individual MAbs followed by incubation with the BLVRcp1 blots and detection using a pool of anti-gp51 MAbs. As predicted, based on our data shown in Fig. 3, none of the neutralizing MAbs prevented gp51 binding to the receptor BLVRcp1. Furthermore, the gp51-MAb complex itself bound to BLVRcp1 (Fig. 4). In these experiments, receptor bound-gp51 was directly detected with secondary anti-mouse AP-conjugate omitting the incubation with the anti-gp51 MAb pool. Immune complexes formed by gp51 and MAbs to neutralizing epitopes F, G, and H bound to 120-kDa MBP-BLVRcp1 were detected by the secondary antibody. The control antibody to p24 did not produce immune complex binding to recombinant receptor (Fig. 4). However, when an anti-gp51 MAb pool was used prior to the secondary antibody the 120-kDa band was observed, confirming the specificity of these experiments (data not shown). Thus, we conclude that virus-neutralizing MAbs were not blocking antibodies and that their neutralizing activity likely involves postbinding events in the process of cell infection. This finding is in agreement with a modeling study suggesting that the three loops in the head of gp51, proposed as the potential interaction site with its cellular receptor, did not contain F, G, and H epitopes (15). Additional postbinding events important for cell infection may include gp51 interaction with other functionally important segments of the receptor, a coreceptor molecule, proteolytic cleavage of the viral glycoprotein (10, 16), or proper exposure of the transmembrane protein gp30, which is required for fusion of virus and cell membrane (39). Similar to those neutralizing BLV, human immunodeficiency virus type 1 (HIV-1)-neutralizing MAbs specific to the gp120 V3 loop effectively blocked cell fusion and virus infectivity independent of gp120-CD4 binding (20). Also, neutralizing MAbs against Epstein-Barr virus prevented virus infectivity independent of receptor engagement (38).

Blocking of BLVRcp1-gp51 binding by polyclonal sera. Bovine sera from BLV-infected or -immunized cattle possess virus-neutralizing activity (2, 4). To determine the mechanism of virus neutralization, bovine sera from seropositive animals naturally infected with BLV were tested for blocking gp51-BLVRcp1 attachment. The anti-gp51 antibody titers of tested sera, determined by enzyme-linked immunosorbent assay (ELISA) (5), are shown in Table 1. Nonspecific binding of bovine sera to MBP-BLVRcp1 lysate was removed by adsorption on Mal-MBP lysate and nondisrupted *E. coli*. Medium containing gp51 was preincubated overnight at 4°C with individual sera followed by incubation with the BLVRcp1 blots and detection of receptor-bound gp51 with a pool of gp51 MAbs and a secondary AP conjugate. Interestingly, in contrast to neutralizing MAbs, preincubation of gp51 with BLV-positive bovine sera prevented gp51 binding to BLVRcp1, as de-

TABLE 1. Antibody titers of bovine sera

Animal no.	Antibody titer	
	ELISA[a]	Blocking[b]
10	3,200	>800
AB1	6,400	200
75	1,600	50
350	3,200	200
750[c]	0	0

[a] The ELISA titer is defined as the inverse value of the last serum dilution at which the optical density in the well with captured gp51 antigen is double that in the well with capturing MAb only.

[b] The blocking titer, determined by receptor binding assay, is defined as the inverse value of the last serum dilution which completely blocks gp51 binding to BLVRcp1.

[c] BLV-noninfected animal.

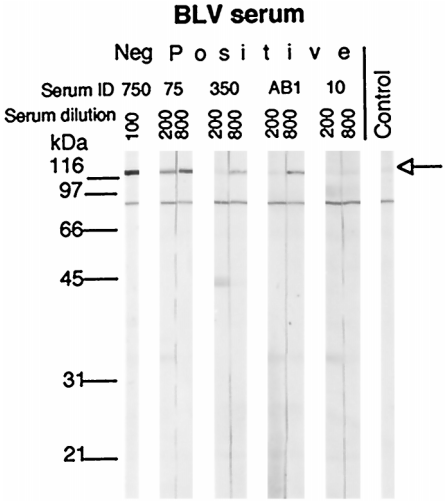

FIG. 5. Bovine serum blocking of gp51 binding to recombinant BLVRcp1. BLV gp51 was preincubated with bovine sera before addition to BLVRcp1 nitrocellulose strips. Detection of gp51 bound to MBP-BLVRcp1 was performed with a pool of anti-gp51 MAbs followed by an AP-conjugated anti-mouse secondary antibody and chromogenic substrate. Serum ID numbers are the same as animal numbers; serum dilutions are given as fold values. The arrow indicates gp51 bound to the 120-kDa MBP-BLVRcp1 protein.

duced by an absence of the 120-kDa band in some samples (Fig. 5). Bovine sera differed remarkably in blocking activity, and the blocking titers ranged from 50 in animal 75 to more than 800 in animal 10 (Table 1). To test the possibility that BLVRcp1-bound gp51 cannot be detected by MAbs (Fig. 5) because bovine antibodies occupied available gp51 epitopes, blots with missing bands were stained with AP-labelled anti-bovine secondary antibody. No bovine antibodies were detected by Western blot, confirming the specificity of the previous assays and the ability of bovine sera to block gp51 binding to receptor (data not shown). BLV-negative sera from animals 833 and 750 did not prevent binding of gp51 to BLVRcp1, and a strong 120-kDa band was detected (Fig. 5, animal 750). Blocking of gp51 binding to BLVRcp1 indicates that BLV-positive sera besides those with antibodies to F, G, and H epitopes (12) contain antibodies recognizing a receptor binding site (hypothesized to be just below the large globular head of gp51, as described in reference 15). Alternatively, these sera may induce conformational changes on gp51 that prevent receptor attachment. Similarly, serum samples from HIV-1-infected humans possess high titers of neutralizing antibodies that block gp120-CD4 binding (17). Since the blocking titers but not the ELISA titers of bovine sera differed greatly among animals, the gp51 binding site most likely is not a prominent antigenic region of gp51, and low-titer sera may not possess blocking activity. In fact, polyclonal sera developed in animals immunized with HIV-1 gp160 peptide displayed potent virus-neutralizing activity but failed to block gp120-CD4 binding (37).

Our preliminary experiments indicate that soluble BLVRcp1 protein blocks infection of OVK, an ovine kidney cell line, and MDBK cells by recombinant BLV (data not shown). Similarly, several soluble viral receptors, such as CD4 for HIV, soluble intercellular adhesion molecule 1 for rhinoviruses, and CD21 for Epstein-Barr virus, have been shown to bind virus and prevent viral infection (13, 23, 26, 40). Further blocking experiments with soluble BLVRcp1 performed with bovine lymphoid cells will address more closely the possibility of an alternative BLV receptor as an explanation for failure of neutralizing MAbs to block gp51 binding to BLVRcp1. Unfortunately, four MAbs prepared to BLVRcp1 did not block gp51 binding to BLVRcp1 (data not shown); however, development of blocking anti-BLVRcp1 MAbs could dissect the possibility that other molecules can participate in BLV attachment in different cell types.

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