Proteins of Bovine Leukemia Virus

I. Characterization and Reactions with Natural Antibodies

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The bovine leukemia virus (BLV) was purified from a chronically infected fetal lamb kidney cell line. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of this virus revealed the presence of eight distinguishable viral components with molecular weights ranging from 80,000 to 11,000. The major component is a non-glycosylated protein having a molecular weight of 24,000 (p24). At least three heavier polypeptides were found, one of them representing a glycoprotein (gp 60). In addition, four minor polypeptides with respective molecular weights of 19,000, 16,000, 13,000, and 11,000 were identified. In a complement fixation assay using naturally occurring antibodies of a leukemic cow, four polypeptides, which included gp 60, p35, p24, and p16, were found to be reactive.

A type C virus, bovine leukemia virus (BLV), is regularly detected in association with bovine leukemia. This agent was described by electron microscopic examination in short-term cultures of lymphocytes from cows with lymphosarcoma as well as with persistent lymphocytosis. Its antigens were characterized by immunodiffusion, immunofluorescence, and complement fixation (CF) using the serum of leukemic or lymphocytotic animals (9, 16-18, 21).

Although considerable information regarding the structural components of other RNA tumor viruses is available (for review, see reference 4), very little is known about the polypeptide content of BLV due to the lack of a permanent source of virus. However, the major internal viral protein has been characterized (10) using continuous producer lines established by the co-cultivation of BLV-producing cells and either bovine embryo spleen or fetal lamb spleen cells (24). We recently obtained from M. J. Van Der Maaten a line of fetal lamb kidney cells chronically infected by the BLV. Its level of production permitted us to study the composition of the virus with regard to its structural proteins and glycoproteins. We report the definition of eight distinct virus components, four of which react with natural antibodies of lymphosarcomatous cow.

MATERIALS AND METHODS

Virus material. BLV was isolated from a fetal lamb kidney cell line provided by M. J. Van Der Maaten. After harvesting, the culture fluids were clarified by filtration through a 0.8- μ m membrane

filter (Millipore), and the virus was concentrated 800-fold by ultracentrifugation at $56,000 \times g$ for 90 min (Beckman rotor 21). After resuspension in a small volume of NTE buffer (0.1 M NaCl, 0.01 M Tris, and 0.001 EDTA at pH 7.4), the pellet was layered on a discontinuous sucrose gradient (15 and 60% in the NTE buffer) and centrifuged for 90 min at $90,000 \times g$ in Beckman rotor 25.1. The opaque band at the interphase was recovered and purified by isopycnic banding on a linear sucrose gradient (15 to 60%). After spinning for 3 h at $130,000 \times g$ in an SW27 rotor, 0.5-ml fractions were collected. A visible band was always found at a density of 1.155 g/cm³ as determined by refractometry. On the average, 0.50 mg of viral protein per liter of culture fluid was obtained.

SDS-polyacrylamide gel electrophoresis. Viral material was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) using a 0.1 M sodium phosphate buffer (pH 7.2) in accordance with the procedure of Weber and Osborn (25). To solubilize viral proteins, 50- μ l samples containing 100 μ g of BLV proteins were boiled for 3 min in 1% SDS and 1% 2-mercaptoethanol. Bromophenol blue was then added, and the samples were applied on 7.5% polyacrylamide gels (0.4 cm/13 cm) containing 0.1% SDS. After electrophoresis at 8 mA/gel until the tracking dye migrated 90% of the gel length, the gels were stained for protein with Coomassie brilliant blue and for carbohydrate with periodic acid-Schiff stain as described by Bolognesi and Bauer (3). Molecular weight markers represented by 20 μ g each of bovine serum albumin (fraction V, Sigma), ovalbumin (grade V, Sigma) and cytochrome c (type VI, Sigma) were treated in a similar way and run in a parallel gel. The molecular weights of BLV proteins were calculated after destaining on the basis of their relative electrophoretic mobility in the SDS-gel preparation.

Elution of BLV proteins. After electrophoresis of a preparation containing 100 μ g of BLV, gels were frozen and sliced into 2-mm fractions with the Bio-Rad gel slicer. Each fraction was eluted by overnight incubation at room temperature in 0.1 ml of 0.1 M phosphate buffer (pH 7.2) with 0.02% SDS.

CF test. The antigenic activity of the different fractions was tested by the CF test using the serum (Se-39) of a lymphosarcomatous cow in accordance with the procedure of P. Boulanger (6). This serum possesses a high level (1:512) of CF antibodies against the antigen prepared as described by Miller and Van Der Maaten (18) (clarified unconcentrated culture fluid of virus-producing cell lines). The CF test was set up with serial twofold dilutions of eluted antigen and an excess (1:64) of the antiserum. The titer of the antigen was taken as the highest dilution that gives 50% hemolysis. Anti-complementary activity of the antigen preparations was also tested, as was their reactivity with a known negative serum from a leukemia-free herd. The molecular weights of the eluted materials registering as positive were determined relative to those of standard proteins and BLV polypeptides. A similar procedure was used for testing the specific antigenic activity of the 1.155-density band of viral preparations.

Radioimmune assay. A 20- μ g portion of purified Rauscher-murine leukemia virus p30 (provided through the auspices of the National Institutes of Health) labeled with 100 μ Ci of 125 I by the chloramine T method was precipitated in an interspecies radioimmune assay using a goat anti-feline leukemia virus serum. Antiserum titration and competitive radioimmunoassay were performed by a double antibody precipitation (20). Competition assays were initiated with 50% of the bound iodinated antigen, and detergent-disrupted BLV was used as the competing antigen. Immune complexes were precipitated with an appropriate titer of a rabbit anti-goat immunoglobulin G serum.

RESULTS

Characterization of purified BLV. The purified particles were identified as BLV on the basis of several physical, biochemical, and immunological criteria. (i) They had a buoyant density of 1.155 g/cm³. (ii) In electron microscopy they were visualized as particles with a 110-nm diameter and a 60- to 70-nm central core separated by a clear area from the external envelope (A. Samso, personal communication). (iii) The reverse transcriptase activity in the presence of synthetic templates required the Mg²⁺ cation (K. Irgens, personal communication). (iv) The preparation had a strong antigenic activity in a CF test that used the serum of a lymphosarcomatous cow (Se-39). (v) There was no cross-reactivity in a radioimmunoassay competition using 125 I-labeled Rauscher-murine leukemia virus p30 precipitated by a goat anti-feline leukemia virus antiserum. This test detects antigenic interspecies determinants that are common to the major internal protein of

murine and feline leukemia viruses shared also by most of the other mammalian type C viruses (22).

Gel electrophoresis analysis of BLV polypeptides. After electrophoresis of the BLV in the presence of SDS and staining with Coomassie brilliant blue, eight major polypeptide bands as well as several additional minor bands were present (Fig. 1). One component was always the most prominent; this major viral polypeptide corresponds to a molecular weight of 24,000 and will be designated BLV p24 according to the current nomenclature of viral proteins (2). Four lower-molecular-weight polypeptides were seen in the electrophoregram: p19, p16, p13, and p11. BLV p13 always appeared as a broad band with a molecular weight varying between 12,500 and 13,000, whereas p16 and p11 were sharper: the p11 band was often irregular, whereas p19 was less abundant than the other three minor polypeptides and its molecular weight ranged from 18,500 to 19,000. In the upper end of the gel, a narrow component having a molecular weight of 80,000 was consistently found. After protein staining, two diffuse bands were also present in the regions that represented molecular weights of 60,000 and 35,000. In fact, these two polypeptides were also detected by other measurements: both of them were defined on the basis of their strong antigenic reactivity (see below), and the polypeptide having a molecular weight of 60,000 was distinguishable due to its carbohydrate content. When the gels were stained with periodic acid-Schiff stain, which detects the presence of glycosylated material (Fig. 2), a distinct band appeared at the region that represented a molecular weight of 60,000. This indicates that the component with a molecular weight of 60,000 that was stained with Coomassie blue corresponds to a glycoprotein which we will designate as BLV-gp 60. There was no other well-defined band after staining with the periodic acid-Schiff stain, although a diffuse red stain was present in the low-molecular-weight part of the gel, probably due to the presence of glycolipids as suggested elsewhere (12).

Several other bands detected with protein staining were not reproducible from experiment to experiment and probably consisted of cellular material.

CF test. BLV antigens eluted from the sliced fractions of the SDS-gel preparation were tested by CF utilizing an excess (1:64) of the immune serum Se-39. Four peaks of CF activity were detected (Fig. 3). The major reactivity (titer 32) was found at the 24,000-molecular-weight level, corresponding to BLV-p24 (Table 1). Two other clearly reactive peaks (titer 8)

FIG. 1. SDS-polyacrylamide (7.5%) gel electrophoresis of 100 μ g of purified BLV. Migration is from top to bottom and staining is with Coomassie brilliant blue.

were detected; one appeared in the region that represented a molecular weight of 60,000 and probably represents the antigenicity of BLV-gp 60. The second, broader reactivity was seen in the region that represented a molecular weight of 35,000, which corresponded to the polypeptide that appeared as a diffuse band in SDS-polyacrylamide gel electrophoresis with protein

staining. A weaker but detectable reactivity was found in the position of p16. No positive reaction was seen in controls with the negative serum of an animal coming from a leukemia-free herd. There was no anti-complementary effect by the eluted antigens.

DISCUSSION

These results show that we were able to define eight different polypeptides of the BLV by using the classical methods for RNA tumor virus protein analysis (4). In addition, an antigenic reactivity with the serum from a diseased animal was found for four of them.

Based on the following morphological and biochemical characteristics, the BLV isolated from a line of fetal lamb kidney cells chronically infected by BLV is identical to BLV from other sources: (i) aspect and size of particles in electron microscopic observations (7, 21, 24); (ii) Mg^{2+} preference of reverse transcriptase (10, 13); (iii) absence of interspecies determinants

FIG. 2. SDS-polyacrylamide gel electrophoresis with periodic acid-Schiff staining. (A) A 100- μ g portion of purified BLV. (B) A 100- μ g portion of purified Rauscher-murine leukemia virus.

FIG. 3. CF activity of BLV polypeptides. The material eluted from 2-mm fractions of SDS gels was tested with a positive serum from a lymphosarcomatous cow (+ + +) and a negative control serum (o o o). Arrows indicate the molecular weight of positive fractions.

common with mammalian type C viruses (8, 10, 15); and (iv) antigenic activity against sera of leukemic cattle (24).

The major protein, BLV p24, was already identified by isoelectrofocusing (10) and is probably identical to the protein having a molecular weight of 25,000 purified from BLV-infected cells (15). BLV-gp 60 has never been described, but a glycoprotein component having a molecular weight of 60,000 was previously isolated by affinity chromatography on concanavalin A Sepharose from culture fluids of fetal lamb spleen cells persistently infected with BLV (J. M. Miller and M. J. Van Der Maaten, *Int. Work. Conf. BLV Infect.*, 2nd, Copenhagen, 1975). Our results suggest that this glycoprotein was probably identical to the gp 60 that we described here. We succeeded in characterizing other polypeptides, p80 and p35, and four low-molecular-weight proteins tentatively designated p19, p16, p13, and p11. However, because SDS-polyacrylamide gel electrophoresis does not constitute a reliable reference method for the determination of molecular weights in this range, further studies using guanidine hydrochloride gel filtration are necessary to determine these more accurately.

Some aspects merit further discussion: gp 60 gives a faint staining in the SDS-gel preparation, but the major glycoprotein is faint in all type C viruses. Moreover, it is well known that the glycoproteins of the murine viruses consti-

tute knobs that are easily released in culture fluids (5) and may be lost during purification procedures (19). The same may be true for BLV, since knobs are loosely bound to the BLV envelope (26) and could explain the large amount of gp 60 which seems to exist in tissue culture fluids (J. M. Miller and M. J. Van Der Maaten, *Int. Work. Conf. BLV Infect.*, 2nd, Copenhagen, 1975). Therefore, it is possible that only small amounts of gp 60 remained on BLV particles at the end of our purification procedures, which explains the relatively weak staining in SDS-polyacrylamide gel electrophoresis. The BLV component having a molecular weight of 35,000, which was also identified on the basis of its antigenic reactivity, may be equivalent to the second viral glycoprotein described in murine leukemia viruses (4) and murine mammary tumor virus (23). However, staining with periodic acid-Schiff stain did not reveal this component in our experiments, probably due to its low carbohydrate content. The signification of p80 remains to be elucidated. Nevertheless, it might represent a precursor of structural polypeptides. A similar component having a molecular weight of 80,000 is found in Rauscher-murine leukemia virus (not shown), which was reported to be the precursor of the major internal protein (1). However, the BLV-p80 does not react against the immune Se-39, whereas p24 does.

Each protein of the BLV was tested with a CF assay against the Se-39 chosen from among 350 sera of animals from leukemic herds (14; D. Levy, L. Deshayes, B. Guillemain, and A. L. Parodi, submitted for publication). It was sampled from a lymphosarcomatous cow 15 days before death and revealed a high level of CF activity when tested against the antigen from the culture fluid of BLV-infected cells. Among the eight described polypeptides, only gp 60, p35, p24, and p16 react with this serum (Table 1). Two antigens are known to react with sera from leukemic cows: p24 (15; J. M. Miller and M. J. Van Der Maaten, *Int. Work.*

TABLE 1. CF activity associated with BLV polypeptides

Designation of polypeptides ^a	Mol wt	CF activity
p 80	80,000	0
gp 60	60,000	++
p 35	35,000	++
p 24	24,000	+++
p 19	18,500-19,000	0
p 16	15,500-16,000	+
p 13	12,500-13,000	0
p 11	11,000	0

^a In accordance with current international nomenclature.

Conf. BLV Infect., 2nd, Copenhagen, 1975) and a glycoprotein having a molecular weight of 60,000 (J. M. Miller and M. J. Van Der Maaten, Int. Work. Conf. BLV Infect., 2nd, Copenhagen, 1975) that probably corresponds to BLV-gp 60. Our method permitted the identification of two other viral antigens, p35 and p16, with p35 demonstrating a particularly strong reaction. Since there are no obvious common antigenic specificities between the different polypeptides of other RNA tumor viruses (11), the four BLV polypeptides probably represent distinct antigens reacting against different antibodies. Nevertheless, further experiments are necessary to test possible cross-reactivities among the four BLV antigens. Monospecific antisera prepared by inoculation of purified proteins will allow a more precise study of these antigens.

Moreover, the significance of the presence of the different antibodies in the natural history of the disease still remains unclear and is under study in our laboratory.

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