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Peripheral Blood Mononuclear Cells from Sheep Infected with a Variant of Bovine Leukemia Virus Synthesize Envelope Glycoproteins but Fail To Induce Syncytia in Culture

ELIZABETH R. JOHNSTON, MAUREEN A. POWERS, LYNN C. KIDD, AND KATHRYN RADKE*

Department of Avian Sciences and Graduate Group in Biochemistry and Molecular Biology, University of California, Davis, California 95616-8532

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Peripheral blood mononuclear cells (PBMCs) infected with the oncogenic retrovirus bovine leukemia virus (BLV) produce virus when cultured briefly. BLV can be transmitted in cocultures to adherent susceptible cells, which become infected, express viral proteins, and fuse into multinucleated syncytia several days later. PBMCs from 3 of 10 BLV-infected sheep displayed a lifelong deficiency in induction of syncytium formation among indicator cells in culture, although large numbers of PBMCs synthesized viral transcripts or capsid protein. Since the infected, syncytium-deficient PBMCs were ≥97% B cells, the deficiency could not be attributed to altered host cell tropism. The syncytium-deficient phenotype was recapitulated in newly infected sheep, demonstrating that this property is regulated by the viral genotype. The alteration in the BLV genome delayed but did not prohibit the establishment of BLV infection in vivo. Envelope glycoproteins were synthesized in syncytium-deficient PBMCs, translocated to the cell surface, and incorporated into virions. However, monoclonal antibodies specific for the BLV surface glycoprotein did not stain fixed PBMCs of the syncytium-deficient phenotype. Moreover, an animal with syncytium-deficient PBMCs had lower titers of neutralizing antibodies throughout the first 5 years of infection than an animal with similar numbers of infected PBMCs of the syncytium-inducing phenotype. The syncytium-deficient variant productively infected indicator cells at greatly reduced efficiency, showing that the alteration affects an early step in viral entry or replication. These results suggest that the alteration maps in the *env* gene or in a gene whose product affects the maturation or conformation, and consequently the function, of the envelope protein complex.

Fusion of viral and cellular membranes by retroviral envelope proteins is essential for viral replication; this capacity for fusion underlies some methods for assessing viral infectivity. Retroviral envelope proteins consist of surface (SU) glycoproteins anchored to the virion membrane by association with transmembrane (TM) proteins. Upon binding of SU to cell surface receptors, the fusion domain of the TM protein adopts a conformation that enables fusion of the viral envelope with the cell membrane (36), which delivers the viral genome-containing core into the cytoplasm of the host cell. After the newly synthesized provirus gains access to host chromatin and is integrated, viral transcripts are synthesized and then translated into proteins that will assemble into progeny virions. Retroviral envelope proteins are initially synthesized as polyprotein precursors that are cleaved during processing through the host cell's secretory pathway; SU and TM remain associated by covalent or noncovalent interactions (reviewed in reference 15). Envelope protein complexes present on the surface of virus-infected cells are incorporated into virions, where they function as oligomers (reviewed in reference 7).

Newly synthesized envelope proteins present on the surface of cells infected by a variety of retroviruses can mediate fusion of the infected cell with other cells displaying appropriate surface receptors (35). The resulting multinucleated giant cells are known as syncytia. Induction of "fusion from within" upon expression of newly introduced virus has been used to quantify cell-free virus, as well as cells that produce infectious virus. On the other hand, viral particles at high concentrations or viral envelope proteins present on the surfaces of infected cells can mediate fusion of cells containing receptors in the absence of further viral replication, a phenomenon termed "fusion from without."

The envelope proteins of the B-lymphotropic retrovirus bovine leukemia virus (BLV) are able to fuse cells in both configurations. Large amounts of cell-free BLV directly fuse susceptible cells to one another without a requirement for new protein synthesis; antibodies specific for SU block this fusion from without (14). Similarly, adherent cells producing BLV quickly induce syncytium formation when susceptible, uninfected cells are added at high density. Monoclonal antibodies specific for the H epitope of BLV SU block this early fusion from without, and neutralizing monoclonal antibodies specific for the F and G epitopes reduce it (6). Feline cells from the CC81 line (13) or its F81 subline (4, 12, 14, 18, 32) are commonly used as adherent indicator cells susceptible to BLV infection and BLV-induced fusion. Cell-free BLV can productively infect these cells, which then fuse with neighboring cells after 5 or more days in culture (4, 23). When peripheral blood mononuclear cells (PBMCs) from BLV-infected cattle and sheep are cocultured with indicator cells, the PBMCs produce virus that infects the indicator cells, which in turn express virus and fuse with neighboring indicator cells (11, 12, 18, 32). If neutralizing antibodies are present while PBMCs produce BLV, later induction of syncytia among indicator cells is greatly reduced, presumably because their productive infection is prevented.

BLV causes tumors in cattle and experimentally infected sheep. An important feature of infection by BLV is that circulating lymphocytes contain few viral gene products, but BLV gene expression can be induced in culture (reviewed in refer-

* Corresponding author. Mailing address: Avian Sciences, University of California, Davis, CA 95616-8532. Phone: (916) 752-9025. Fax: (916) 752-8960. Electronic mail address: klradke@ucdavis.edu.

ence 17). We have shown that PBMCs from a group of BLV-infected sheep displayed two distinctive patterns of BLV expression (18). For two-thirds of the animals, substantial numbers of PBMCs produced viral transcripts and induced syncytia in culture. In contrast, only a few PBMCs from the remaining animals induced syncytium formation among indicator cells, even though a large number of cells contained viral RNA by in situ hybridization. Here, we show that cells from the latter animals presented a lifelong pattern of deficient syncytium induction that did not change over the course of infection despite fluctuations in the number of BLV-infected cells. The syncytium-deficient phenotype was recapitulated in newly infected sheep, indicating that we have identified a genetic variant of BLV. The mutation(s) in the BLV genome delayed but did not prohibit the establishment of BLV infection in vivo. PBMCs infected with this BLV variant generated virus that failed to support productive infection of the indicator cells used in our infectious center assay. We present evidence suggesting that the mutation lies in the *env* gene or in a gene whose product affects the maturation or conformation, and consequently the function, of the envelope protein complex.

MATERIALS AND METHODS

Infected animals. Sheep 167, 407, 409, 410, 468, and 469 had been infected by either subcutaneous or intraperitoneal injection of 8×10^6 BLV-Bat$_2$ cells and culture supernatant containing 2.5×10^4 syncytium-inducing units of cell-free BLV as previously described (18, 24). Four newly acquired sheep were infected with 9×10^5 capsid (CA) protein-positive PBMCs obtained from two of the previously infected sheep: sheep 212 and 213 were injected with 12.8×10^6 PBMCs from sheep 407, while sheep 214 and 215 were injected with 8.6×10^6 PBMCs from sheep 468. Two control sheep were injected with 12.8×10^6 cells from uninfected sheep 471. Before being injected, PBMCs that had been frozen without being cultured were thawed, diluted in Iscove's modified Dulbecco medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), and cultured overnight at 37°C. After being centrifuged and resuspended in 1 ml of Iscove's modified Dulbecco medium–10% FBS, the cells were injected intradermally into each recipient at three or four sites on the neck. In addition, 5 ml of medium from the appropriate overnight culture was injected into the jugular vein.

Preparation of PBMCs. Blood was collected from BLV-infected sheep by venipuncture into acidic citrate-glucose. PBMCs were prepared by centrifuging cells from buffy coats over Histopaque-1077 (Sigma) as previously described (18). Cells were washed twice before being cultured.

Syncytium assays. Adherent F81 cells that form syncytia when infected with BLV were used as indicator cells as described previously (24). Duplicate samples of PBMCs (2.5×10^5 or fewer cells per ml) were cultured in 22-mm-diameter wells with indicator cells in liquid growth medium (minimal Eagle's medium supplemented with 5% FBS) or in growth medium containing 1% (wt/vol) methylcellulose. PBMCs and indicator cells were cocultured for 48 h, and then PBMCs were washed off the monolayers; indicator cells were fed fresh medium and cultured for 3 more days. After the indicator cells were fixed and stained with Giemsa, syncytia containing more than five nuclei were counted at a magnification of ×100.

Neutralizing antibodies. Antibodies that neutralized the infectivity of cell-free virus obtained from BLV-Bat$_2$ cells were measured by the ability to prevent late syncytium induction among F81 indicator cells, as previously described (23). The neutralizing titer is the reciprocal of the serum dilution yielding a 50% decrease in the number of syncytia.

In situ hybridization of BLV RNA. Samples of 4×10^5 PBMCs were cultured for 2 to 5 h at room temperature in Iscove's medium–10% FBS, deposited onto slides with a cytocentrifuge, and then fixed as previously described (17). Viral RNA was hybridized to ^{35}S-labeled antisense RNA probes prepared from five subclones that spanned the genome of the T15-2 clone of BLV (10), and the preparations were subjected to autoradiography with nuclear track emulsion. Cells scored as positive had more than five times the number of silver grains found as background over other cells in the field.

Blot hybridization of BLV transcripts. Cytoplasmic RNA was prepared from cultured PBMCs by homogenization in buffered 0.65% Nonidet P-40 as previously described (22). Poly(A)$^+$ RNA was selected by adsorption to oligo(dT)-cellulose and quantitated by spectroscopy. Samples were separated by electrophoresis through a 1% agarose–2.2 M formaldehyde gel. After transfer onto a nylon membrane, transcripts were hybridized with a ^{32}P-labeled RNA probe complementary to the *tax*/*rex* region of the BLV genome. The washed blot was exposed to Kodak XAR-5 film with an intensifying screen at −80°C.

Blot hybridization of BLV provirus. DNA obtained from noncultured PBMCs was digested three times with *Hin*dIII or *Sac*I at 5 U/μg of DNA as previously described (24). Each lane of a 0.8% agarose gel contained 12 μg of DNA. Nylon membranes were baked for 30 min at 80°C, and then DNA was covalently linked to the membrane by UV (120,000 μJ/cm^2; Stratalinker). Prehybridization was overnight at 42°C in 6× SSC (0.9 M NaCl, 90 mM Na citrate)–0.6% sodium dodecyl sulfate (SDS)–50 μg of herring sperm DNA per ml. For use as a probe, 25 ng of the 8,095-bp *Sac*I fragment of the T15-2 tumor clone of BLV was radiolabeled to a specific activity of 1×10^9 to 3×10^9 dpm/μg by extension of random hexamers (Prime-It RmT Kit; Stratagene) and 200 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). The probe (2×10^7 cpm) was hybridized for 48 h at 42°C in 50% formamide–6× SSC–0.6% SDS–50 μg of herring sperm DNA per ml. Excess probe was washed from the membrane; the most stringent wash was for 30 min at 41°C in 0.1× SSC–0.5% SDS. Membranes were exposed to Kodak XAR-5 film between two intensifying screens at −80°C.

Immunocytochemistry. Numbers of PBMCs containing BLV CA protein were determined by immunocytochemistry as previously described (16). PBMCs were cultured at 5×10^5/ml for 20 h in the presence of 10 μg of lipopolysaccharide (LPS; from *S. minnesota*; Sigma) or 2.5 μg of phytohemagglutinin L (PHA-L; Sigma) per ml of medium. The cells were then deposited onto slides and fixed first with 4% paraformaldehyde and then with acetone. Fixed cells were incubated with a 1:500 dilution of rabbit anti-CA serum (16), with a 1:100 dilution of a biotinylated goat secondary antibody, and then with biotinylated alkaline phosphatase linked to avidin. Sites of antibody binding were visualized by using a HistoMark Red substrate (Kirkegaard & Perry Laboratories); cell nuclei were counterstained with 0.5% methyl green. CA-positive cells and the total number of cells were counted at a magnification of ×200 or ×400.

Immunofluorescence. To stain SU, PBMCs were cultured and fixed onto slides as described above. Fixed cells were first incubated for 2 h at room temperature with a mixture of monoclonal anti-SU antibodies (directed against epitopes A, B, B′, C, D, D′, E, F, G, and H; reference 5) diluted 1:50 in blocking buffer (5% nonfat milk, 25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.075% Tween 20). After each incubation, slides were washed three times in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.4). Samples were then incubated for 1 h at room temperature with a biotinylated sheep anti-mouse antibody diluted 1:1,000 with PBS containing 0.1% azide and 0.1% bovine serum albumin (PBS-AB). Finally, samples were incubated for 45 min in the dark with phycoerythrin-streptavidin (Cytochemical kit; Amersham) diluted 1:50 in PBS-AB.

For simultaneous staining of the light chains of surface immunoglobulin (Ig) and CA protein, PBMCs were cultured overnight and then incubated in PBS-AB with a 1:100 dilution of fluorescein-conjugated F(ab′)$_2$ fragments of a donkey antibody specific for the heavy and light chains of sheep IgG. After the cells had been washed and deposited onto slides with a cytocentrifuge, they were fixed, permeabilized, and incubated with rabbit anti-CA serum. The secondary antibody was a rhodamine-conjugated goat antibody specific for the F_c portion of rabbit IgG. To visualize nuclei, cells were stained for 10 min with 5 μg of Hoechst 33258 per ml of PBS. Preparations were counted at a magnification of ×400 under UV illumination by using a UV-, fluorescein-, or rhodamine-selective filter.

Radiolabeling and immunoprecipitation. PBMCs (5×10^6/ml) were cultured for 4 h in complete medium (Iscove's modified Dulbecco medium supplemented with 7.5% FBS) containing 2.5 μg of PHA-L per ml. Cells were then pelleted by centrifugation at 300 × *g*, resuspended at 6.7×10^6/ml in cysteine- and methionine-deficient Dulbecco's modified Eagle's medium (ICN Biomedicals) supplemented with 7.5% FBS, 15 μg of L-methionine per ml, and 2.5 μg of PHA-L per ml, and then returned to the flask. [^{35}S]cysteine (130 to 170 μCi/ml; 1,300 Ci/mmol; Amersham) was added, and the cells were incubated for 8 h. Labeled cells were pelleted at 110 × *g* and washed three times in cold PBS before being resuspended in 2 ml of lysis buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [wt/vol] Nonidet P-40, 1% [wt/vol] sodium deoxycholate, 2 mM EDTA, 1 mg of ovalbumin per ml, 1% [vol/vol] aprotinin, 100 μg of phenylmethylsulfonyl fluoride per ml) and incubated on ice for 20 min. Radioactive culture medium was centrifuged at 3,000 × *g* for 5 min to remove any cells remaining. The supernatant was then centrifuged at 47,000 × *g* for 15 min to pellet virions, which were then lysed in 1 ml of lysis buffer as described above.

After being cleared of insoluble material by centrifugation at 47,000 × *g* for 30 min, lysates of cells (2 ml) and virions (1 ml) were each incubated at 4°C for 4 to 20 h with 4 μl of normal rabbit serum that had been premixed with 50 μl of 50% (wt/vol) protein A-Sepharose in lysis buffer and kept for 1 h at 4°C. After removal of beads and bound proteins, lysates were immunoprecipitated serially with a rabbit anti-CA antibody and then with either a rabbit anti-SU antibody (27) or mouse monoclonal anti-SU antibodies (specific for linear epitopes A, B, B′, C, D, and D′). At each step, either 4 μl of antiserum or 4 μl of mixed monoclonal antibodies premixed with 50 μl of 50% (wt/vol) protein A- or protein G-Sepharose was added to cleared lysates and the mixtures were incubated at 4°C for 4 to 20 h. After each round of immunoprecipitation, pellets were washed twice in lysis buffer and then once in 20 mM Tris-HCl (pH 8.0). Each SU pellet was divided into two portions, and one was treated with N-glycosidase to remove N-linked carbohydrate as described below. SU proteins were resolved on SDS–7.5% polyacrylamide gels, whereas CA proteins were resolved on SDS–10% polyacrylamide gels. Gels were fixed in 30% methanol–10% acetic acid, impregnated with En^{3}Hance (Du Pont), dried, and then exposed to Kodak XAR-5 film at −80°C. Intensities of bands on the autoradiograms were quantitated with a

Bio-Image densitometer and software (Millipore). Several different exposures were analyzed for each experiment to ensure that values were in the linear range.

N-glycosidase digestion. SDS (10% wt/vol) was added at 0.1 volume to the pellet of proteins adsorbed to washed protein A-Sepharose beads, and the mixture was boiled for 2 min. After a 10-fold excess of lysis buffer was added to reduce the concentration of SDS to 0.1%, the mixture was boiled again for 2 min. N-glycosidase F (Boehringer Mannheim) was added at 1 U/ml of the lysate originally precipitated, and the mixture was incubated at room temperature for 3 h. SDS sample buffer (double strength: 125 mM Tris-HCl [pH 8.4], 4% [wt/vol] SDS, 20% [wt/vol] glycerol, 0.01% [wt/vol] bromophenol blue, 100 mM dithiothreitol) was then added in a volume equivalent to that of the beads, and the sample was boiled. Beads were pelleted at 16,000 × *g* in a microcentrifuge, and the supernatant was collected and applied to a gel.

Biotinylation of cell surface proteins. PBMCs (4×10^6/ml) were cultured for 10 to 12 h with 1.25 μg of PHA-L per ml, harvested and washed once with PBS, and then suspended at 10^7/ml in PBS. Biotin (0.1 to 0.2 mg of LHS-biotin; Pierce) was dissolved in 100 μl of PBS just before being added to the cell suspension to a final concentration of 50 to 100 μg/ml. After incubation at room temperature for 15 min or at 4°C overnight, the reaction was stopped by adding 5 ml of buffer (20 mM Tris-HCl [pH 7.4], 5 mM glycine, 140 mM NaCl, 10 mM EDTA; reference 29) to each sample and the mixture was incubated at room temperature for 10 min. Cells were centrifuged at 300 × *g* and washed once with Tris-glycine buffer. The cell pellet was then lysed and immunoprecipitated with a polyclonal anti-SU antibody as described above for radiolabeled protein.

After being separated on SDS-polyacrylamide gels, proteins were transferred onto Hybond-ECL membrane (Amersham). The gel was first equilibrated in running buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 30 min. Electrotransfer was at 20 V overnight at room temperature or at 60 to 70 V for 3 h at 4°C. Biotinylated SU protein was visualized by using streptavidin-linked horseradish peroxidase at a dilution of 1:1,000 to 1:2,000 and enhanced chemiluminescence (ECL kit; Amersham). Blots were exposed to Hyperfilm-ECL (Amersham) for 2 to 30 min, and band densities were quantified by densitometry as described above.

RESULTS

Identification of a persistent syncytium-deficient phenotype. When the ability of PBMCs to synthesize BLV RNA or to act as infectious centers is assessed, only a few rare infected cells express virus in vivo but many more cells carrying BLV proviruses can readily be detected after short-term culture (17, 18, 23). We have reported that the initial period of BLV infection in sheep is marked by dramatic and transient increases in the number of PBMCs that can express BLV when cultured briefly (18, 23). Analysis of viral transcripts by in situ hybridization of PBMCs cultured for 3 to 5 h revealed that all animals had large numbers of BLV-positive cells for a short period following initial infection (Table 1, early peak). Most animals also had concurrent increases in the number of PBMCs that acted as infectious centers by transmitting BLV to indicator cells, which fused into multinucleated syncytia after 5 days in culture. However, PBMCs from animals 468 and 469 induced very small numbers of syncytia despite the fact that large numbers of PBMCs transcribed BLV. During the first year after infection, animal 167 also had very few PBMCs that acted as infectious centers in culture; we did not assess its BLV-transcribing cells until later. For these three animals, the initial deficiency in infectious center activity persisted throughout the first 2 years after infection (Table 1) although the total number of BLV-infected PBMCs, measured by number of cells that expressed BLV RNA, fluctuated over time and reached levels as high as or higher than those from other BLV-infected animals.

Later during infection, we measured the capacity of PBMCs to synthesize viral proteins in culture. The number of PBMCs that synthesized BLV CA protein after 20 h in culture was determined by immunocytochemical staining with a polyclonal antibody (16). CA-positive PBMCs fluctuated in number during the course of infection in both sets of animals, as illustrated by the data obtained from two animals between 4 and 7 years after infection (Fig. 1). Of the PBMCs from sheep 407 that synthesized CA protein, 7 to 50% induced syncytia in the infectious center assay. In contrast, only 0.02 to 4% of CA-positive PBMCs from sheep 468 induced syncytia. Comparison of the maximum number of syncytia induced by PBMCs at any time during their lifelong infection with the number of PBMCs that were capable of synthesizing CA protein on the same day (Table 2) demonstrates that BLV-infected PBMCs from animals 167 and 468 were greatly deficient in syncytium induction but were able to support viral protein synthesis. Thus, animals 167, 468, and 469 carried lifelong BLV infections in which PBMCs displayed a syncytium-deficient phenotype. Moreover, syncytia that did form were smaller than those initiated by PBMCs with a syncytium-inducing phenotype.

B-cell hosts for BLV. BLV infects predominantly B cells (reviewed in reference 28), so a difference in the lineage of the host cell for BLV in syncytium-deficient PBMCs might provide a basis for understanding this phenotype. To investigate this issue, PBMCs from animals 407 and 468 were cultured overnight and stained as live cells with fluorescein-conjugated $F(ab')_2$ fragments of antibody to detect light chains of sheep Ig. After being fixed and permeabilized, cells were incubated with anti-CA antibodies and then with a rhodamine-conjugated secondary antibody. In repeated experiments, ≥97% of the CA-positive PBMCs had the light chains of sheep Ig on their surface. Thus, B cells were the predominant hosts for BLV among both syncytium-inducing and syncytium-deficient PBMCs.

Transmissibility of the syncytium-deficient phenotype. To determine whether the difference in syncytium induction by PBMCs from the two sets of animals was due to a genetic difference in the virus, rather than to an unusual interaction of

TABLE 1. Numbers of PBMCs positive for BLV RNA and syncytium induction during the first 2 years after infection[a]

Animal no.	Early peak[b]		Year 1[c]		Year 2[d]	
	No. of RNA$^+$ cells	No. of syncytia (ratio)[e]	No. of RNA$^+$ cells	No. of syncytia (ratio)	No. of RNA$^+$ cells	No. of syncytia (ratio)
407	2,183	81 (27)	167	22 (8)	909	37 (25)
409	440	137 (3)	120	55 (2)	440	156 (3)
410	4,119	166 (25)	—[f]	—	—	—
167	—	2	—	7	437	9 (49)
468	3,740	8 (468)	745	3 (248)	983	2 (492)
469	1,250	14 (89)	117	3 (39)	527	6 (88)

[a] Data are numbers of RNA-positive cells or syncytia per 500,000 PBMCs. Cells were cultured in medium containing FBS.
[b] Data (from reference 18) represent averages of five measurements beginning with the onset of high expression, between 15 and 63 days after infection.
[c] Data are averages of five measurements during days 344 to 489.
[d] Data are averages of two to four measurements during days 728 to 887.
[e] Ratio of RNA$^+$ cells to syncytia.
[f] —, not tested.

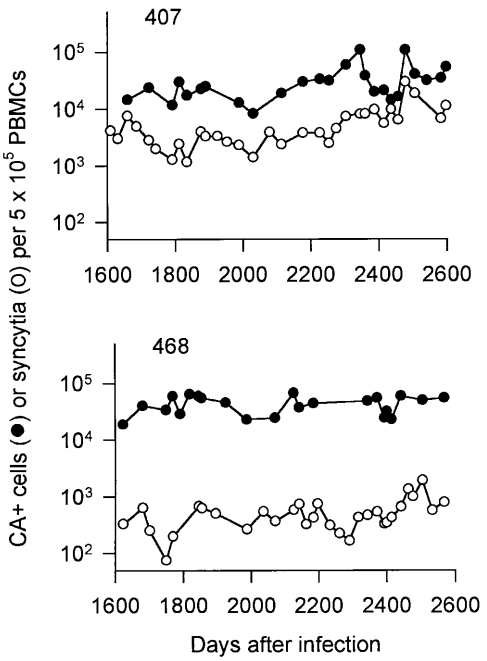

FIG. 1. Relative levels of syncytium-inducing PBMCs in animals 407 and 468 between 4 and 7 years after infection. PBMCs were cultured overnight in the presence of 10 μg of LPS per ml, and the frequency of CA-positive cells was determined by immunocytochemical staining. In parallel, PBMCs suspended in 1% methylcellulose medium containing LPS were cocultured in duplicate with indicator cells for 2 days. The average number of syncytia that developed among the indicator cells after a total of 5 days is plotted for each time point. All values are adjusted to represent the number of positive cells per 5×10^5 PBMCs.

BLV with cells from particular animals, we infected four new sheep. Two were infected with cells from animal 407, whose PBMCs had normal syncytium-inducing capacity, and two others were infected with cells from animal 468, whose PBMCs had low syncytium-inducing capacity. All four were injected with 9×10^5 CA-positive PBMCs. The cells chosen as inocula for the new animals were obtained in year 7 of infection of animals 407 and 468. DNA from these PBMCs was analyzed by Southern blot hybridization and was found in each case to exhibit polyclonal proviral integration sites with some oligoclonal expansion of BLV-infected cells (data not shown). PBMCs were collected from the newly infected animals twice weekly and were assessed for CA protein expression by immunocytochemistry analysis and for syncytium induction by an infectious center assay.

PBMCs collected from the two sets of newly infected sheep differed in syncytium induction (Fig. 2). Quantitation of

TABLE 2. Lifetime maximum number of syncytium-inducing PBMCs with concurrent number of CA-positive cells

Animal no.	No. of positive cells/500,000 PBMCs[a]	
	Syncytia	CA protein (ratio)[b]
407	33,910	45,800 (1.4)
409	17,850	9,012 (0.5)
410	66,400	210,000 (3.2)
167	856	11,986 (14.0)
468	1,936	50,000 (25.8)

[a] Cells were cultured with 10 μg of LPS per ml.
[b] Ratio of CA^+ cells to syncytia.

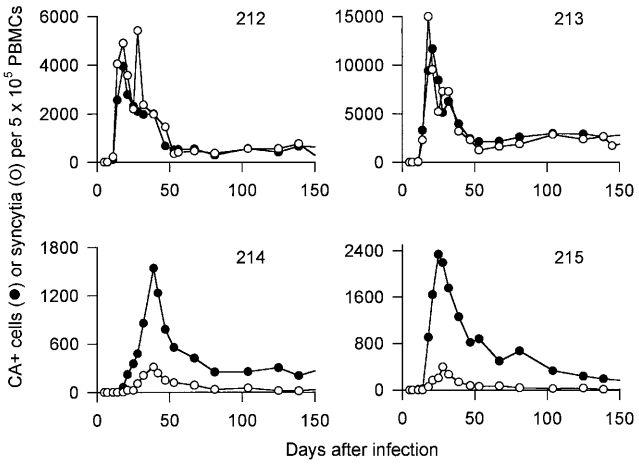

FIG. 2. Transmission of the syncytium-inducing and syncytium-deficient phenotypes to newly infected sheep. Animals 212 and 213 were injected with 9×10^5 CA-positive, syncytium-inducing PBMCs from animal 407; in parallel, animals 214 and 215 were injected with the same number of CA-positive, syncytium-deficient PBMCs from animal 468. Cells from the newly infected animals were tested at regular intervals for the ability to synthesize CA protein after being cultured overnight in medium supplemented only with serum and for the ability to induce syncytia after being cocultured with indicator cells in methylcellulose medium.

PBMCs that acted as infectious centers was done in medium containing methylcellulose, a syncytium assay whose efficiency was greater than that of the liquid culture assay used for the initial group of infected animals. Cells from sheep (no. 212 and 213) inoculated with normal BLV-infected PBMCs included large numbers of CA-positive cells and induced large numbers of syncytia among indicator cells. During the first 150 days of infection, the number of CA-positive cells approximately equaled the number of syncytia formed. In contrast, cells from sheep (no. 214 and 215) infected with syncytium-deficient PBMCs recapitulated the phenotype of animal 468, since only 1/5 to 1/11 of the CA-positive cells induced syncytia in the infectious center assay. These results indicate that the syncytium-deficient phenotype was transmitted by one or more mutations in the BLV genomes present in PBMCs from animal 468.

This mutation appeared to cause a short delay in the establishment of infection in vivo. CA-positive cells from the two animals infected with BLV from animal 407 reached their highest levels 18 days after infection. In contrast, sheep infected with syncytium-deficient BLV from animal 468 attained peak numbers of CA-positive cells at 25 and 39 days after infection. We initially had observed delays of 4 and 9 weeks in peak numbers of RNA-positive cells in animals 469 and 468 (18). These delays may be related to the deficiency in syncytium induction in culture.

Lower titers of neutralizing antibodies. Syncytium induction in culture is mediated by the interaction of retroviral envelope proteins with cellular receptors. One indicator of viral envelope protein synthesis in vivo is the presence of host antibodies, including neutralizing antibodies, that are specific for envelope proteins. To determine whether envelope proteins of the syncytium-deficient PBMCs might be displayed differently or at low abundance in vivo, we measured titers of neutralizing antibodies. Dilutions of serum were tested for the ability to reduce syncytium induction by cell-free BLV obtained from the same cell line as the virus used to inject the initial group of sheep. Animal 468, whose cells had the syncytium-deficient

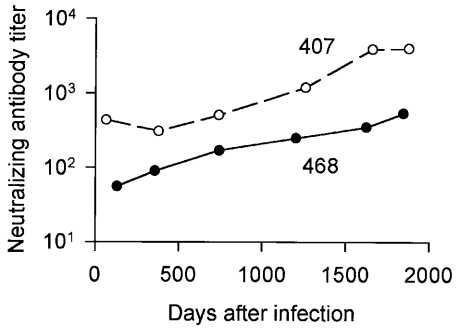

FIG. 3. Strength of virus-neutralizing antibodies in sera obtained from animals 407 and 468 during the first 5 years after infection. Titer represents the reciprocal of the serum dilution that elicited 50% inhibition of infection of indicator cells by cell-free BLV.

phenotype, had up to 10-fold lower titers of neutralizing antibodies during the first 5 years after infection than animal 407, whose cells induced syncytia normally (Fig. 3). Throughout this period, these two animals had comparable levels of PBMCs that transcribed BLV RNA or synthesized BLV CA protein in short-term culture (Table 1 and unpublished data), so their apparent levels of infected PBMCs did not differ greatly although the syncytium-inducing capacities of the cells did. Thus, BLV did not elicit neutralizing antibodies as effectively in the animals whose PBMCs had the syncytium-deficient phenotype as the virus in animals with syncytium-inducing PBMCs.

Synthesis and location of SU glycoprotein. We performed a series of experiments to determine whether viral *env* mRNA was transcribed and translated in syncytium-deficient PBMCs of animal 468 and whether the envelope glycoproteins were located on the surfaces of infected cells. Analysis of polyadenylated transcripts from syncytium-deficient PBMCs indicated that BLV envelope mRNA was present, had the expected size of 4.4 kb, and was transported from the nucleus to the cytoplasm as required for translation (22). To detect transcripts, we used a radioactive probe complementary to the *tax*/*rex* region of the BLV genome. Since this region is present in all major BLV transcripts, the intensity of hybridization is proportional to the amount of a transcript present. Figure 4 shows that the ratios of *gag-pol* to *env* transcripts were similar in the cytoplasms of PBMCs that were syncytium inducing (animal 407) and syncytium deficient (animal 468).

Since there was no gross deficiency of *env* mRNA in syncytium-deficient cells, we investigated the amount of envelope protein synthesized. PBMCs from animals 410 and 468 were used in these experiments, since the frequencies of CA-positive cells in these two animals were most similar at the time. PBMCs were cultured for a total of 12 h in medium containing PHA-L to stimulate viral expression. [^{35}S]cysteine was added to the cultures after 4 h, a time when cytoplasmic *env* mRNA is abundant and PBMCs start to become CA positive (22). Detergent lysates were then subjected to two sequential rounds of immunoprecipitation, first with a polyclonal antiserum directed against the CA protein and then with a polyclonal antiserum directed against the SU surface glycoprotein. To enable resolution of SU as a well-defined polypeptide, half of the immunoprecipitates were also treated with N-glycosidase to remove carbohydrate prior to electrophoresis. To monitor virion production and protein content, lysates of virions isolated from the culture medium were examined in the same manner.

PBMCs from both animals synthesized large amounts of CA protein that was immunoprecipitated from lysates of both cells and virions (Fig. 5A). As expected, the Gag-Pro and Gag polyprotein precursors containing CA determinants were immunoprecipitated from cell lysates. (The slight difference in mobility of the Gag precursors from 468 PBMCs was not reproducible.) SU was also present in lysates of both PBMCs and virions (Fig. 5B). The SU protein was detected in lysates of primary PBMCs only after treatment with N-glycosidase to remove carbohydrate, since the glycosylated form of SU migrated on gels as a smear between 50 and 60 kDa in an area with a high background level. Deglycosylated SU polypeptides migrated as a well-defined band of 30 kDa that comigrated with similarly treated polypeptides from a BLV-producing cell line (data not shown); monoclonal antibodies specific for SU recognized the 30-kDa polypeptides from both sources. Analysis of the culture medium by immunoprecipitation after virions were removed showed that some soluble SU protein was released by PBMCs from each animal, but that there was no preferential shedding by the syncytium-deficient cells (data not shown). This suggests that the TM protein was present and that SU remained associated with it (33). These results indicate that CA and SU proteins were synthesized by syncytium-deficient PBMCs and incorporated into virions released into the culture medium. The presence of SU in virions indicated that SU was properly localized at the plasma membrane of syncytium-deficient PBMCs because the viral envelope is derived from the cell surface during the budding of virions.

Autoradiograms were analyzed by densitometry to compare the amounts of CA and SU proteins. After the twofold difference between the two animals in the number of CA-positive PBMCs and the underloading of one lane of the gel were accounted for, cells from animal 410 that initiated syncytium formation at high frequency synthesized twice as much of the CA and SU proteins per infected cell as the syncytium-deficient cells from animal 468. CA and SU protein levels were also twofold higher in virions produced by the syncytium-inducing cells than in virions produced by the syncytium-deficient cells. Since the levels of both CA and SU proteins differed in parallel, there was no selective deficiency of envelope

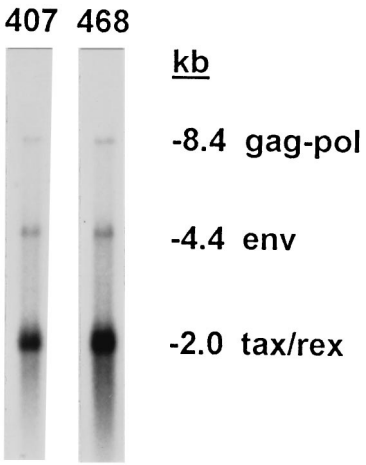

FIG. 4. Cytoplasmic BLV transcripts in syncytium-inducing and syncytium-deficient PBMCs. PBMCs (1.2×10^8) obtained from animals 407 and 468 during year 5 after infection were cultured at 10^6/ml in medium supplemented with 10 μg of LPS per ml. After 10 h, cells were lysed by homogenization in buffered detergent. Poly(A)$^+$ RNA (1 μg per sample) was analyzed on a Northern (RNA) blot by using a ^{32}P-labeled antisense *tax*/*rex* probe. The blot was exposed to film for 48 h. Sizes of BLV transcripts are indicated. Animal 468 had twice as many CA$^+$ PBMCs as animal 407 at the time.

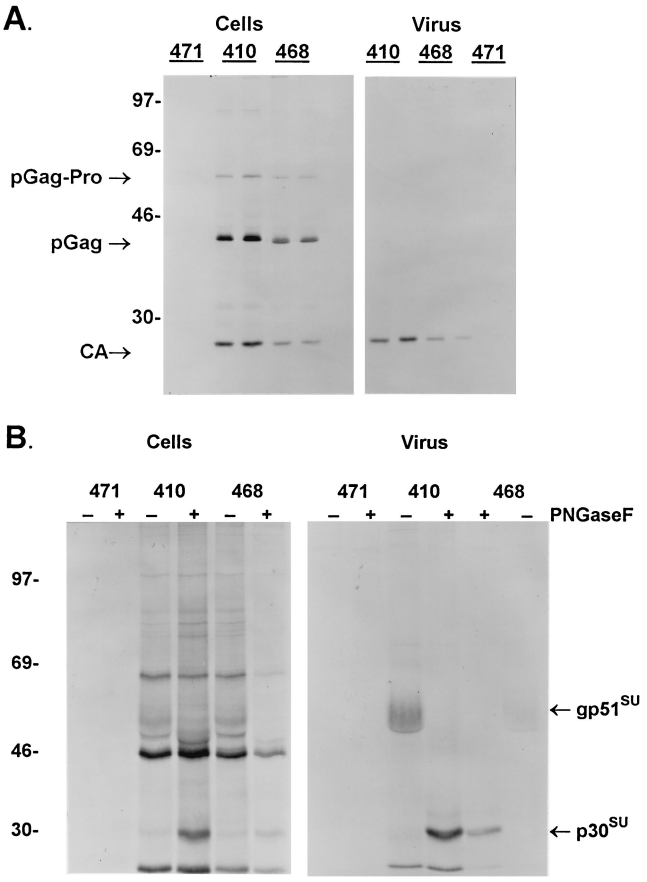

FIG. 5. CA and SU proteins synthesized by syncytium-inducing and syncytium-deficient PBMCs. PBMCs from BLV-infected animals 410 and 468 and from uninfected control animal 471 were cultured for 12 h in the presence of 2.5 μg of PHA-L per ml. Proteins synthesized between 4 and 12 h were labeled with [^{35}S]cysteine. Detergent lysates prepared from cells or virions recovered from the culture medium were serially immunoprecipitated with rabbit polyclonal antibodies specific for CA (A) and SU (B). Each lane on the polyacrylamide gel contains protein immunoprecipitated from 40×10^6 cells or from virions produced by this number of cells. Samples immunoprecipitated with anti-SU were either left untreated (−) or treated (+) with peptide N-glycosidase F (PNGaseF) to enable the resolution of SU as a 30-kDa polypeptide. Gels were exposed to film for 26 h (CA), 5 days (SU, cells), and 21 days (SU, virus). BLV proteins are indicated on the autoradiograms, as are the positions of marker proteins (molecular masses are in kilodaltons).

protein in syncytium-deficient cells or virions. The twofold lower amounts of CA and SU in virions produced by PBMCs from animal 468 contrast sharply with the 62-fold lower level of infectious center activity measured concurrently with this labeling experiment. This pattern was reproduced in two additional experiments.

The fact that SU was incorporated into virions suggested that this protein was located at the cell surface. To compare the amount of SU present on the plasma membranes of the PBMCs directly, we derivatized cell surface proteins with biotin. SU protein was immunoprecipitated with anti-SU polyclonal serum, treated with N-glycosidase, resolved by SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. Biotinylated SU was detected by enhanced chemiluminescence with a streptavidin-horseradish peroxidase conjugate. Densitometric analysis of autoradiograms revealed twice as much SU on the surfaces of syncytium-inducing PBMCs from animal 410 as on those of syncytium-deficient PBMCs from animal 468 (data not shown), indicating that SU protein was present on the cell surface in direct proportion to its concentration in cellular lysates. Thus, SU was proportionate in abundance to CA protein in cultured PBMCs from animal 468, as determined by both immunoprecipitation of radiolabeled SU from lysates of cells and virions and by direct analysis of SU on the surface of PBMCs.

Finally, we performed immunocytochemistry analysis of fixed PBMCs from animals 410 and 468 by using anti-CA serum or a mixture of monoclonal antibodies specific for SU (Table 3). Whereas CA-positive cells exceeded SU-positive cells 4-fold in animal 410, they were 83-fold more abundant than SU-positive cells in animal 468. In both animals, the percentages of SU-positive and syncytium-inducing cells agreed closely. Thus, in contrast to the ability of polyclonal antibodies to bind SU in solution, the monoclonal antibodies failed to bind SU on fixed cells from animal 468; this failure correlated with the inability of these cells to induce syncytium formation. These results suggest that the SU glycoprotein has a different conformation in BLV-infected PBMCs of animal 468 and that the *env* gene contains one or more mutations.

Syncytium-deficient PBMCs fail to establish productive infection of indicator cells. Since the SU glycoprotein was synthesized, directed to the cell surface, and incorporated into virions produced by PBMCs with the syncytium-deficient phenotype but seemed to have a different conformation, we wanted to determine whether the virus produced by these PBMCs failed to establish an infection in indicator cells or whether there might be a later defect in syncytium induction by infected indicator cells. We monitored synthesis of CA protein within indicator cells as a mark of successful infection, reasoning that a mutation blocking early events in infection would block synthesis of structural proteins, as well as subsequent syncytium formation, whereas a mutation that affects syncytium formation itself would yield a large number of CA-positive indicator cells but few syncytia. To accommodate the different conditions for assessment of CA synthesis and syncytium formation, we titrated indicator cells with overlapping ranges of PBMCs. This provided quantitative results within the linear range for each of the two assays. We cocultured PBMCs with indicator cells for 1 day, washed off the PBMCs, and fixed some of the indicator cells on day 3. These were immunostained for CA protein. The rest were fixed on day 5 and stained with Giemsa for counting of syncytia. PBMCs cultured in parallel were immunostained after 20 h to calculate the number of CA-positive PBMCs that had actually been seeded onto indicator cells.

As shown in Fig. 6, the number of indicator cells that were CA positive on day 3 correlated well with the number of syncytia formed by day 5, whether PBMCs were of the syncytium-inducing or of the syncytium-deficient phenotype. Once an indicator cell proceeded to the point of synthesizing CA protein, it could participate in syncytium induction. A duplicate

TABLE 3. Deficiency of SU-positive cells determined by immunostaining[a]

Animal no.	% of cells		
	CA^+	SU^{+b}	Synctium inducing
410	33	12	8.8
468	12	0.15	0.16

[a] Cells were cultured for 20 h with 10 μg of LPS per ml.
[b] Cells were stained with a monoclonal antibody mixture.

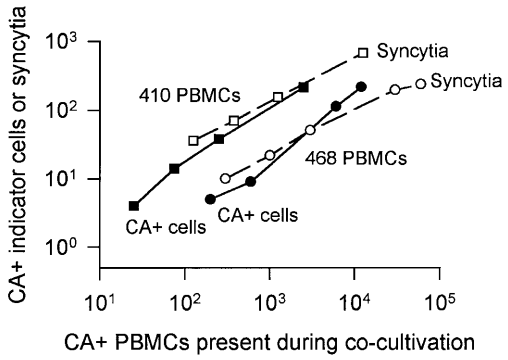

FIG. 6. Infection of indicator cells and induction of syncytium formation among indicator cells by syncytium-inducing PBMCs from animal 410 and syncytium-deficient PBMCs from animal 468. PBMCs were suspended in methylcellulose medium containing 10 μg of LPS per ml and seeded into 0.75-cm^2 chambers of glass slides that had been preplated with 1.8×10^4 indicator cells or into 3.8-cm^2 wells that had been preplated with 9×10^5 indicator cells for the usual infectious center assay. The number of PBMCs seeded per well was titrated through a range that was dictated by the size of the well and the estimated frequency of CA^+ PBMCs. (The lowest number was 10^2 cells per well for animal 410, and the highest was 3×10^4 cells per well for animal 468.) After 1 day, PBMCs were washed off and the indicator cells were fed fresh medium. Two days later, the indicator cells on chamber slides were fixed and immunostained for CA protein. Indicator cells in large wells were fixed 4 days later and stained with Giemsa, and syncytia were counted. The actual frequency of CA^+ PBMCs present in these samples was determined by immunostaining of PBMCs cultured in liquid medium for 20 h. Results are presented as numbers of CA^+ indicator cells (closed symbols) or syncytia (open symbols) obtained per calculated number of CA^+ PBMCs present during cocultivation with indicator cells.

experiment performed 1 month before this one gave a similar pattern of results. Thus, virus produced by PBMCs of the syncytium-deficient phenotype either failed to enter indicator cells or failed to replicate at some early point before synthesis of structural proteins of the virion.

DISCUSSION

The deficiency of syncytium-inducing PBMCs during initial infection of a group of BLV-infected sheep was quite striking. Since the phenotype was recapitulated in newly infected animals, a variant viral genotype was responsible. To have affected so many of the initial group of animals, this variant must have been encoded by a provirus in the BLV-infected bat cell line that served as the source of the original inoculum. All animals were probably injected with a mixture of syncytium-inducing and variant genomes. The variant was not outgrown by syncytium-inducing virus in the three animals with syncytium-deficient PBMCs, so it did not seem to be at a selective disadvantage in vivo. The delayed appearance of variant-infected PBMCs in circulating blood during initial infection was noteworthy. This delay, together with the lower titer of neutralizing antibodies in an animal infected with the syncytium-deficient variant, suggests that the variant had an effect in vivo and that deficiency in syncytium induction was not solely an in vitro phenomenon.

The inability of the BLV variant to induce syncytium formation in culture differs in several respects from the extensively characterized syncytium-inducing properties of human immunodeficiency virus type 1. Non-syncytium-inducing variants of human immunodeficiency virus type 1 are found among virus isolates from infected individuals (3, 8, 25, 26, 31). These do not induce syncytia among primary PBMCs, whereas syncytium-inducing variants do. Non-syncytium-inducing human immunodeficiency viruses can be isolated during all stages of infection, are monocyte tropic, and do not replicate in T-cell lines. Syncytium-inducing viruses are more frequently isolated as infection progresses to disease and replicate well in T-cell lines. In contrast, we have not observed syncytia among cultured PBMCs from any BLV-infected sheep. Rather, we used syncytium induction as a tool to assess BLV infectivity. Host cell tropism did not differ among syncytium-inducing and syncytium-deficient PBMCs: B cells were the major hosts for BLV. Furthermore, the deficiency in syncytium-inducing PBMCs was a lifelong phenomenon in three of the originally infected animals.

All of the originally infected animals that had syncytium-deficient PBMCs eventually developed tumors, which occurred at 4 (no. 469) and 8 (no. 167 and 468) years after infection (16). Of the seven animals with syncytium-inducing PBMCs, three developed tumors at 1.5 (no. 169; reference 17), 6 (no. 410), and 9 (no. 410) years after infection. The other four animals with syncytium-inducing PBMCs were euthanized because of failure to thrive (at 1 year for sheep 470; reference 17), paralysis (at 4 years for sheep 380), wasting (at 7 years for sheep 381; unpublished data), or bacterial endocarditis (at 8 years for sheep 409). Since tumors occurred between 1.5 and 9 years after infection and all animals but one that were euthanized for other reasons were terminated within that time span, the BLV variant that fails to induce syncytium formation may predispose the host to eventual tumor formation. Learning about tumorigenesis in the sheep newly infected to test transmissibility of the syncytium-deficient phenotype will help to clarify this issue.

The syncytium-inducing or non-syncytium-inducing phenotype maps to the part of the human immunodeficiency virus *env* gene encoding the SU glycoprotein, including sequences encoding variable loops V2 and V3 and areas that may interact with those regions (1, 2, 30). The syncytium-deficient phenotype may map in the *env* gene of BLV as well. Failure of monoclonal antibodies against eight different epitopes of SU to bind fixed, syncytium-deficient PBMCs, despite the fact that SU protein could be immunoprecipitated from these cells, supports the notion that a mutation in *env* may alter the conformation of the envelope protein complex.

The amino acid sequence of the BLV envelope proteins is remarkably conserved among isolates from different geographical areas (19) and within an individual animal during asymptomatic infection and tumorigenesis (37, 38). This conservation can probably be attributed to several factors. First, the functional properties of envelope proteins of human T-cell leukemia virus type I, a close relative of BLV, appear to be highly constrained by the protein sequence (21); nonconservative substitutions of amino acids in a number of locations impair protein processing or syncytium induction (9). Second, the reverse transcriptase of BLV has a low error rate (20). Third, oligoclonal expansion of infected cells may be an important route of viral propagation, as suggested by results obtained with individuals infected with human T-cell leukemia virus (34). Proofreading by cellular DNA polymerases would reduce the introduction of errors into the provirus during the S phase of cell division.

The defect in syncytium induction by the BLV variant interfered with infection before synthesis of viral CA protein in indicator cells. If the sequence of one of the envelope proteins is altered, this must affect binding to the cellular receptor or fusion of the viral membrane with the host cell membrane. A search for altered *env* sequences is under way. If the alteration is in another gene, its product may interfere with the correct configuration of the envelope proteins or with some step in viral replication preceding the late stage of gene expression. In

that case, determination of the step that is blocked in replication would provide clues to the identity of the gene.

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