Transcriptional Activation of Bovine Leukemia Virus in Blood Cells from Experimentally Infected, Asymptomatic Sheep with Latent Infections

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Infection by bovine leukemia virus (BLV) is characterized by a long latency period, after which some individuals develop B-cell tumors. The behavior of BLV and related retroviruses during the latency period between initial infection and subsequent tumorigenesis is poorly understood. We used in situ hybridization to detect BLV transcripts in individual peripheral blood mononuclear cells from experimentally infected, asymptomatic sheep with latent infections. Viral RNA was not found in most peripheral blood cells that had been isolated as rapidly as possible from circulating blood, but it was present in rare cells. BLV RNA transcripts increased in a biphasic manner within a few hours after the blood cells were placed in culture. Exposure to fetal bovine serum was identified as the principal cause of this transcriptional activation, which occurred in fewer than 1 in 1,000 cells. Agents known to activate immune cells polyclonally caused a further increase in the number of cells containing BLV RNA within 8 h. In some cases, the numbers of viral transcripts within individual cells also increased. Thus, BLV is not detectably expressed in most resting lymphocytes circulating in the blood, but its transcription is activated by components of fetal bovine serum and can be augmented by molecules that mimic activation of immune cells. This activation, which might occur in lymphoid tissue during an immune response, may lead to the synthesis of viral regulatory proteins that are thought to initiate tumorigenesis through host cell genes.

Bovine leukemia virus (BLV), human T-cell leukemia virus types I and II (HTLV-I and HTLV-II, respectively), and human immunodeficiency virus (HIV) are all retroviruses that replicate in cells of the immune system. Infection by these viruses is followed by a long latency period, and a certain proportion of infected individuals then rapidly progress to terminal disease. Because these viruses integrate essentially at random into the host genome and do not contain cell-derived oncogenes, it is thought that the viral gene products themselves lead to development of disease. The genomes of BLV, HTLV, and HIV encode *trans*-acting factors that regulate viral transcription, but little is known about how viral transcripts that encode these regulatory proteins are initially induced. For HIV, recent evidence suggests that immune responses to unrelated antigens trigger virus replication and contribute to disease progression (reviewed in reference 15).

BLV, HTLV-I and HTLV-II are tumorigenic retroviruses that cause leukemia and lymphoma (reviewed in references 4 and 53) and are distinct from HIV, a lentivirus that causes fatal immunodeficiency and neuropathy of the central nervous system (reviewed in reference 15). BLV and HTLV-I share numerous features of infection and pathology. They also have similar genomic structures (43) and use common strategies for gene expression. The 3' ends of the genomes of HTLV and BLV contain the X sequences encoding a set of novel proteins (30, 39, 41, 45) that are necessary for efficient transcription from the viral promoter (7, 11, 40) and that determine the relative proportions of spliced and unspliced transcripts (12, 25, 27). Aside from host range, a notable difference between the two viruses is that BLV infection is associated with malignancy of B cells, whereas HTLV affects T cells.

Tumor cells contain at least part of one provirus, but the viral genome is not detectably expressed (16, 28), which suggests that viral gene products are not necessary to maintain the transformed state. The X proteins encoded by the virus may set in motion a series of events leading to secondary genetic changes that result in malignant transformation (54). It is important for two reasons to know where and under what conditions viral genes are expressed before tumor development begins. (i) Intermittent production and spread of the virus would favor transformation simply by increasing the population of cells at risk for such a genetic change. (ii) It is possible that the X-encoded proteins, tax and rex, could be produced from a subgenomic message without production of virions and that tax itself is responsible for changes in cells that could lead to uncontrolled growth (19, 26, 33).

BLV-specific RNAs have not previously been detected in RNA extracted from populations of mononuclear cells newly purified from the blood of infected animals (20, 29), but BLV transcripts were detected once these cells had been in short-term culture (20). However, low-level transcription in a few cells might escape detection unless individual cells were examined for viral transcripts. Virion proteins and viral particles have been detected in a few cells as early as 3 to 6 h after initiation of blood cell cultures but were most abundant after 24 to 48 h of culture (3, 14, 49).

To examine infected cells during the latency period, when virus expression is likely to be important in disease development, we studied transcriptional activation of the BLV provirus in peripheral blood mononuclear cells (PBMCs) from infected but asymptomatic sheep that had no elevation of lymphocyte counts. We have found that the number of cells that act as infectious centers in culture is extremely low during latency, typically about 1 in 10^4 cells (submitted for publication). Here, the single-cell sensitivity of in situ hy-

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bridization allowed us to examine and measure viral RNA levels in PBMCs from animals with latent BLV infections.

We found that rare mononuclear cells rapidly isolated from peripheral blood contained a low amount of BLV RNA but that most infected cells did not contain detectable viral RNA. Removal from the blood and subsequent culture resulted in BLV RNA production in as many as 1 in 2,000 cells; BLV transcripts increased in a biphasic manner within these cells. Fetal bovine serum (FBS) present in the culture medium was identified as the principal inducer of this transcription. Further treatment with certain polyclonal activators of immune cells increased not only the number of cells containing BLV RNA but also the level of viral transcripts within each cell.

MATERIALS AND METHODS

Blood cells and plasma. The blood cells used for the work described in this paper were taken from sheep beginning 1 year after experimental infection with BLV (manuscript submitted). Blood was collected from the jugular vein into acid citrate glucose (used as an anticoagulant; Becton Dickinson Vacutainer Systems, Rutherford, N.J.). Buffy coats from 20 ml of blood were harvested from collection tubes by centrifugation at 1,200 × g for 10 min, diluted with 10 ml of Hanks balanced salt solution without Ca^{2+} and Mg^{2+}, and layered onto 5 ml of Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.), and mononuclear cells were separated from neutrophils, eosinophils, and remaining erythrocytes by centrifugation at 1,200 × g for 30 min. Cells at the interface were rinsed three times with Hanks balanced salt solution without Ca^{2+} and Mg^{2+} and suspended to 2 × 10^6 cells per ml in Iscove modified Dulbecco medium (IMDM) supplemented with 1× nonessential amino acids, 2 mM L-glutamine, 100 μg of streptomycin per ml, 100 U of penicillin per ml, 5 × 10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated FBS, except when otherwise noted. These PBMCs were either harvested immediately or cultured under various conditions in a humidified 7% CO_2, 37°C incubator. The mitogens or activators used were lipopolysaccharide (LPS; from *Salmonella minnesota*; Sigma), phytohemagglutinin (PHA-P; Difco Laboratories, Detroit, Mich.), pokeweed mitogen (PWM; from *Phytolacca americana* tuber; Sigma), concanavalin A (ConA; Sigma), and rabbit anti-sheep immunoglobulin M (Organon Teknika, Malvern, Pa.).

For plasma, 10 ml of blood was collected into 143 U of heparin (Sigma) and the tubes were centrifuged as described above. The supernatant was centrifuged at 500 × g for 3 min and then at 10,000 × g for 5 min to remove residual platelets and used immediately without storage.

Attachment of cells to glass slides. Slides were acid washed, treated with Denhardt solution, and acetylated as previously described (22) to minimize nonspecific absorption of the probe and maximize retention of the cells. The PBMCs (5 × 10^5) were suspended in IMDM–10% FBS and deposited onto these slides in a Shandon Cytospin II cytocentrifuge at 300 rpm for 10 min. The slides were quickly dried with a stream of cool air, and the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline containing 10 mM $MgCl_2$ for 10 min (31). The slides were transferred directly from formaldehyde to 70% ethanol and stored at 4°C.

Construction of probe templates. A plasmid containing BLV DNA isolated from a bovine tumor (13) was used to make the probe templates. Five fragments approximately 1 to 2 kilobases long that together spanned the BLV genome were directionally cloned into pGEM-3 and pGEM-4

(Promega Biotec), derivatives of pSP64 and pSP65 (35) that contain a multiple cloning site flanked by promoters for the SP6 and T7 bacteriophage RNA polymerases. The subclones contained a 1,688-base-pair (bp) SacI-BamHI fragment encoding the *gag* region and half of the protease gene, a 1,916-bp HincII-BamHI fragment containing the last third of the protease gene and the reverse transcriptase-encoding segment of *pol*, a 1,493-bp BamHI-HincII fragment encoding the endonuclease segment of *pol* and most of the gp60 portion of *env*, a 1,633-bp HincII-BamHI fragment containing the gp30 portion of *env* and the region upstream of *X*, and a 1,238-bp BamHI-SacI fragment containing the *X* coding regions.

The plasmids were linearized by restriction enzymes that do not leave 3' overhangs (44) and that cleave within the multiple cloning site at the 3' end of the BLV sequences. Linearized templates were extracted with phenol-chloroform, harvested by ethanol precipitation, and washed with 70% ethanol containing 0.2 M sodium acetate, pH 6. These DNAs served as templates for in vitro transcription. Probes transcribed from these templates were tested for their sequence specificity by Southern analysis and for strand specificity on dot blots containing RNAs from fetal lamb kidney (FLK) (52) and Bat_2clone_6 (18) cells, which express BLV, and from an uninfected parent bat cell line, Tb_1Lu.

Radioactive labeling of single-stranded RNA probes. Single-stranded RNA probes were labeled with ^{35}S-labeled ribonucleoside triphosphates to a specific activity of 10^8 to 10^9 cpm/μg essentially as recommended by Promega Biotec, the supplier of the pGEM vectors. A 20-μl volume of the reaction mixture contained the following, added in order at room temperature: 5× transcription buffer (200 mM Tris hydrochloride [pH 7.5], 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 1 U of RNasin (Promega Biotec) per μl, 500 μM each GTP and CTP, 12.5 μM each UTP and ATP, 50 μCi each of [^{35}S]UTP (1,200 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and [^{35}S]ATP (1,000 Ci/mmol; Amersham), 800 ng of template DNA, and 15 U of T7 RNA polymerase (Promega Biotec). Dithiothreitol was not added, because the amount contained in the ^{35}S-labeled ribonucleotide solution brought its final concentration to almost 10 mM. After 1 h of incubation at 37°C, 1 U of RNase-free DNase (RQ DNase; Promega Biotec) was added and the incubation was continued for 10 min. RNA was extracted with phenol-chloroform and then with chloroform and concentrated with 2.5 volumes of ethanol after addition of sodium acetate (pH 5.2) to 0.25 M.

The probes were reduced in length by partial alkaline hydrolysis (9). From 1 to 5 μg of the labeled RNA probe was taken up in 150 μl of 40 mM $NaHCO_3$–60 mM Na_2CO_3 (pH 10.2) and hydrolyzed at 60°C for 10 min. Ten minutes of hydrolysis was chosen as optimal after determination of the signal-to-noise ratios of probe samples hydrolyzed for various times. After hydrolysis, the solution was neutralized by addition of 7.5 μl of 2 M sodium acetate, pH 6, and 7.5 μl of 10% acetic acid and then precipitated with 2.5 volumes of ethanol. The labeled probes were stored at −20°C under ethanol until just before use.

Hybridization. Slides removed from storage in ethanol were rehydrated in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) for 15 min and treated before hybridization as previously described (24). Hybridization was performed with minor modifications (9). The hybridization mixture contained the radiolabeled probe (approximately 2 × 10^6 cpm per cytodot; 2 ng/μl) in 0.5 mg of salmon sperm DNA per ml–0.75 mg of yeast tRNA per ml–0.1 mg of poly(A) per

ml–50% formamide–10% dextran sulfate–0.3 M NaCl–20 mM Tris hydrochloride (pH 7.6)–5 mM EDTA (pH 8)–1× Denhardt solution–10 mM dithiothreitol.

Cells were deposited on slides within a circle 6 mm in diameter. To cover the samples, 22-mm^2 siliconized cover slips were baked at 250°C for 4 h to ensure removal of RNases and scored diagonally and broken just before use. A 7.5-µl volume of hybridization mixture was applied to each cytodot, which was covered with a siliconized half cover slip, and the edges were sealed with a generous portion of rubber cement. The slides were kept at 50°C on a slide warmer for 4 h (9). The rubber cement was carefully removed with forceps, and the slides with the cover slips were placed in a Coplin jar containing 50% formamide–2× SSC prewarmed to 50°C (24). After 10 min, the cover slips were gently teased off and the slides were transferred to a second Coplin jar containing 50% formamide–2× SSC at 50°C for an additional 10 min. After a brief rinse in 2× SSC, the slides were transferred to 2× SSC containing 20 µg of RNase A (Sigma) per ml and 100 U of RNase T$_1$ (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml at 37°C for 30 min. The slides were washed in 2× SSC (100 ml per slide) at 50°C for 1 h and in 0.1× SSC at 50°C for 30 min, dehydrated in graded ethanols, and stored at room temperature until coated with emulsion.

Autoradiography. Slides were coated with NTB2 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.) diluted 1:1 with water, dried horizontally for 30 min, and then transferred to a slide dryer (Oncor) for 30 min. Dry slides were sealed in black plastic slide boxes containing desiccant. The boxes were kept at 4°C until ready to develop, usually after 12 h of exposure.

Developer, rinse, and fix solutions were pre-equilibrated to 15°C. The slides were equilibrated to room temperature for 30 min and then developed for 3 min in D-11 developer (Eastman Kodak), rinsed for 30 s in 1% acetic acid, fixed for 3 min in rapid fixer (Eastman Kodak), and rinsed for 5 min in water. While in developer and fixer, the slides were agitated at 30-s intervals. Thoroughly dry slides were stained with a commercial Wright-Giemsa-type stain (Diff-Quik; American Scientific Products, McGaw Park, Ill.).

Quantitation of BLV RNA by grain counts. Silver grains that developed in the nuclear track emulsion were counted under a 60× or 100× planapochromat oil immersion objective of a Nikon photomicroscope. Grains were counted over each positive cell among the 5×10^5 cells contained within the cytodot (6 mm in diameter). The background was determined by counting the number of grains over the cells in 10 randomly selected fields; the mean background varied from 0.2 to 2 grains per cell.

Preliminary experiments to estimate the copy number indicated that the number of viral RNAs represented by grains developed after 12 h of exposure to the emulsion was approximately two genomic-length copies per grain. The stably infected FLK cell line (52), which contains an average of 3,000 full-length copies of viral RNA per cell (2), was used as a standard. Grains over FLK cells were reduced to a countable number by lowering the specific activity of the probe, and then grains formed after 4, 8, and 12 h of exposure to the emulsion were counted to determine the reduction in grains that was effected by reducing the exposure time. Finally, FLK cells that had been hybridized to a probe with the usual specific activity were exposed to the emulsion for 4 h and the numbers of grains counted were extrapolated to those that would have developed after 12 h of exposure.

RESULTS

BLV RNA-containing PBMCs found in freshly isolated cells from BLV-infected sheep. PBMCs were obtained from sheep experimentally infected with BLV. PBMCs freshly purified from the blood of these BLV-infected animals were analyzed by in situ hybridization with antisense RNA probes spanning the entire BLV genome and were found to contain BLV RNA. The frequency of viral-RNA-containing cells ranged from 1 in 2,000 cells to fewer than 1 in 500,000 cells, varying in each animal at different times after infection. Numbers within this range are characteristic of the latency period of virus infection in animals (manuscript submitted). This asymptomatic state lies between the initial establishment of infection and the later appearance of disease symptoms.

Representative samples of the rare cells expressing BLV RNA are illustrated in Fig. 1. Silver grains indicate the presence of viral transcripts in a cell with lymphocyte morphology (Fig. 1A) amid freshly prepared PBMCs. Figure 1B shows how the level of viral RNA became elevated after the cells were cultured for 6 h. Cells with monocyte morphology occasionally also contained BLV RNA (Fig. 1C), but cells with typical lymphocyte morphology were by far the most common BLV RNA-containing cells. Monocytes have not been described as hosts for BLV. Since there are no antibodies specific for sheep monocytes, our assignment of some of the relatively rare BLV-positive cells to the monocyte lineage was based on morphology alone.

Biphasic increase in BLV RNA in PBMCs from BLV-infected animals during short-term culture. BLV RNA has not been detected in PBMCs from infected animals until the cells have been cultured (21, 28). This is at odds with our finding of BLV RNA in uncultured blood cells (Fig. 1). We reasoned that the viral RNA we had detected in freshly prepared blood cells would not have been observed in previous studies if the number of BLV transcripts had been too low or if the BLV RNA had been present in too few cells to be detected. It was possible that we had identified rare BLV RNA-containing cells because of the single-cell sensitivity of in situ hybridization. Alternatively, the PBMCs might have begun to synthesize viral RNA during the interval between blood collection and fixation of cells on slides; this was up to 6 h. By the time cells were sampled for in situ hybridization, they had been kept at room temperature in medium containing 10% FBS for up to 3 h.

We first compared PBMCs that were rapidly isolated and fixed within 2 h of bleeding with those prepared during routine separation. The grains present over rapidly isolated cells were reduced in number and located primarily over the nuclei, suggesting that they represented newly synthesized RNA. To estimate the amount of RNA contained within infected cells in vivo, we measured BLV RNA in cells fixed at various intervals after their removal from the animals and extrapolated back to the time when the blood was drawn.

Experiments with four different animals showed that viral transcripts were either extremely rare or nonexistent in populations of infected PBMCs prepared as rapidly as possible after the bleeding of an animal (Fig. 2). A constant number of cells representing those that most actively transcribed the viral genome was selected for analysis in each experiment, and transcriptional activity was averaged. BLV RNA appeared slowly during a lag that lasted for several hours, and then transcript levels increased rapidly. In early samples, grains were situated over the nuclei (as in Fig. 1A), and in later samples they were also located over the cytoplasm (as in Fig. 1B). Time points beyond 14 h were not
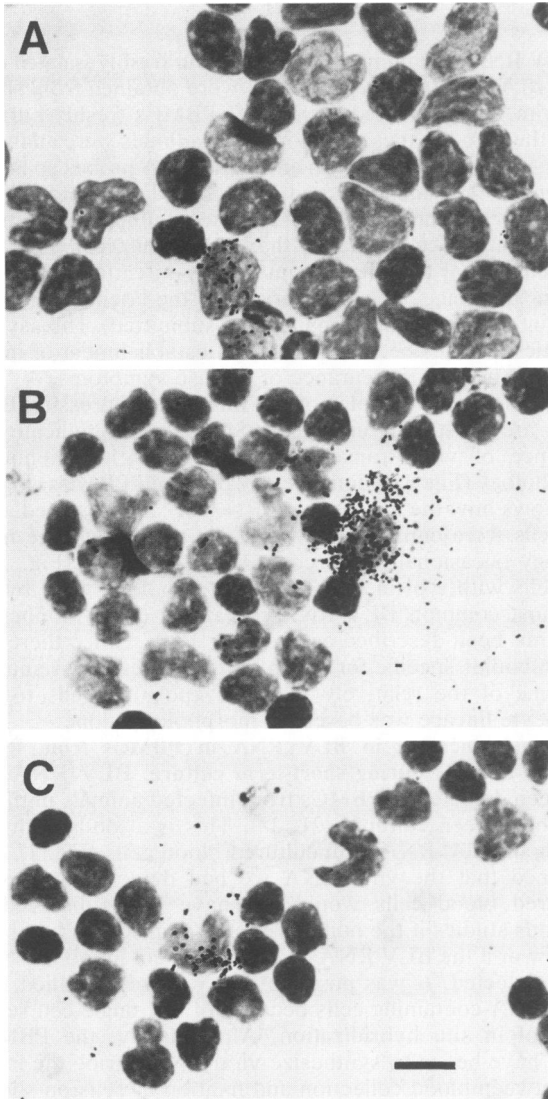
FIG. 1. Detection of BLV transcripts in PBMCs. ^{35}S-labeled BLV antisense RNA probes were hybridized to RNA in fixed mononuclear cells isolated from the blood of BLV-infected sheep, and slides were exposed to emulsion for 12 h. Silver grains are located over a BLV RNA-positive cell in each panel. (A) Freshly prepared, uncultured cells. (B) Cells after 6 h in culture. (C) A positive cell with monocyte morphology. Bar, 10 μm.
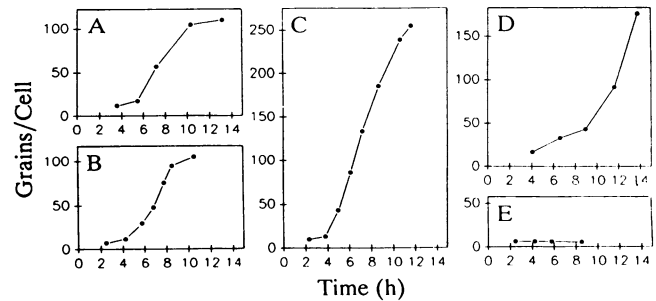

FIG. 2. Kinetics of appearance of BLV transcripts in PBMCs after their removal from the animal. PBMCs were rapidly isolated from the blood and kept at 37°C in culture medium containing 10% FBS. At specified intervals, cell samples were removed for quantitation of BLV RNA by in situ hybridization. Grains over positive cells in individual samples of 5×10^5 cells were counted and averaged. Time is given in hours from the time of blood sampling. The cells used for these experiments were taken from sheep that had been infected with BLV for at least 1 year and at times when 0.004 to 0.02% of the PBMCs of a particular animal were capable of producing virus in an infectious-center assay. The results of experiments performed on different days with cells from four infected animals are presented. Panels: A, animal 468 at 12 months postinfection; B, animal 380 at 28 months postinfection; C, animal 409 at 28 months postinfection; D, animal 407 at 13 months postinfection; E, animal 471, an uninfected control.

included, since BLV RNA might have begun to appear in secondarily infected cells. Since the plots of grains per positive cell extrapolate essentially to zero at the time of bleeding, these data suggest that for animals in latency, BLV transcription does not take place in most infected cells while they circulate in the peripheral blood.

However, our analysis of transcription in single cells revealed that rare cells from rapidly processed samples of blood contained moderate amounts of BLV RNA. For example, among the PBMCs fixed by 2.3 h after the bleeding of animal 409 for the experiment shown in Fig. 2, five cells contained 20 to 31 silver grains. No cells from the uninfected animal contained these numbers of grains. Thus, a few cells contained a moderate amount of BLV RNA when examined as soon as possible after the bleeding of animals.

Significant increase in transcription of BLV RNA caused by an event after removal of cells from blood. Time course experiments showed that production of BLV transcripts began sometime during or after removal of blood from an animal. We wanted to know whether this expression of the virus was induced simply by the act of collecting blood or whether expression was induced by transfer of the blood from body temperature to ambient laboratory temperatures.

To test whether viral transcription was triggered by perturbation of the natural PBMC environment, we performed the following experiment. Blood was collected and kept at body temperature from the moment of bleeding. Another sample was collected at ambient temperature as usual. After the blood was kept for 6 h at these temperatures, PBMCs were rapidly isolated and fixed on slides. We compared the presence of viral RNA in (i) cells kept in blood or (ii) PBMCs isolated immediately upon our return from bleeding the animals. Half were fixed without delay, and half were placed in culture with 10% FBS for 6 h.

One cell in three samples of 5×10^5 PBMCs rapidly isolated from three different animals contained 34 silver grains (Table 1). Again, a rare cell from an infected animal contained a moderate amount of BLV RNA when chemically fixed after having been purified from the blood as rapidly as possible. When citrate-treated blood was kept for 6 h at either 22 or 37°C, 1 to 4 cells in each sample of 5×10^5 PBMCs contained viral RNA. These few cells may have been activated in the collection process but not because of the temperature shift. The larger number of grains over cells from blood kept at 37°C may be explained by a higher transcription rate at the physiological temperature. In contrast to the small number of BLV RNA-containing cells found among cells kept in blood, 26 to 160 in 5×10^5 cells contained BLV transcripts when kept in culture for an equivalent time. Thus, neither the change in temperature nor collection alone could account for the induction of viral transcription observed in culture.

BLV expression in mononuclear cells of BLV-infected sheep

TABLE 1. BLV RNA in cells kept in blood[a]

Cell treatment	No. of positive cells/5 × 10^5 cells (avg no. of grains/ positive cell)		
	Animal 167	Animal 380	Animal 409
None (purified cells before culture)	1 (34)	0	0
37°C, 6 h, IMDM–10% FBS	26 (64)	160 (112)	41 (177)
37°C, 6 h, citrate-treated whole blood	1 (138)	3 (81)	1 (32)
22°C, 6 h, citrate-treated whole blood	ND[b]	4 (28)	ND

[a] The experiments were performed on three different days.
[b] ND, Not done.

not prevented by autologous plasma. BLV-infected PBMCs reportedly do not produce virus when cultured in 100% plasma from BLV-infected animals (20), and evidence for a transcriptional inhibitor has been presented (21). Although the results of our blood storage experiments may be interpreted to mean that BLV expression is inhibited in blood, we were unable to block the appearance of BLV RNA in PBMCs by culturing these cells in autologous plasma. Freshly isolated PBMCs were rinsed free of platelets by centrifugation through FBS and then cultured in 100% autologous plasma for 6 to 24 h. In 17 different experiments, we found BLV RNA in these cells at levels equivalent to those found in individual cells under ordinary culture conditions, and most of the time, the number of cells that produced viral RNA was also equivalent.

Prompted by the report of a platelet-derived factor that activates BLV transcription, even in the presence of 100% plasma from BLV-infected animals (51), we repeated our experiments and omitted the rinse with FBS. Great care was taken during isolation of PBMCs to remove platelets gently without lysing them and to use platelet-cleared autologous plasma to wash residual platelets from the PBMC preparation. Despite these measures, some platelet lysis must have occurred because we observed some small clots upon microscopic inspection of stained slides. The results of experiments on cells from two animals are presented in Table 2. The numbers of cells containing BLV RNA in samples cultured in 100% autologous plasma were reduced to 17 to 36% of the numbers found in samples cultured in medium with 10% FBS. However, the average amount of RNA per positive cell was not greatly affected by culturing the cells in plasma. Since cell viability was sometimes compromised by culture in plasma, we tried to separate the effects of culture conditions from those of an inhibitor of BLV transcription. The inhibitor of BLV transcription that has been found in

TABLE 2. BLV RNA in PBMCs cultured in autologous serum or plasma

Culture medium[a]	No. of positive cells/5 × 10^5 viable cells (avg no. of grains/positive cell)	
	Animal 380	Animal 407
IMDM–10% FBS	479 (147)	215 (125)
IMDM–10% autologous serum[b]	311 (180)	182 (76)
100% Autologous serum[b]	332 (199)	62 (126)
100% Autologous plasma	174 (117)	37 (122)

[a] PBMCs were cultured for 9 h at 37°C.
[b] Sera were heated for 30 min at 56°C before use.

plasma from BLV-infected cattle is not present in their sera (20), so we cultured PBMCs from our infected sheep in 100% autologous serum. The numbers of cells with BLV RNA in these samples were reduced to 29 to 69% of the numbers found in samples cultured in medium with 10% FBS, but cell viability was still compromised. PBMCs cultured in medium with 10% autologous serum were healthier, and BLV RNA-containing cells were present at 65 to 85% of the numbers in medium with 10% FBS. Thus, while examining BLV transcription in PBMCs found at low frequency in the blood of sheep with latent BLV infections, we were unable to demonstrate a plasma-specific block to BLV transcription. Perhaps the clotting of residual platelets in the cell preparations induced normal levels of transcription in a reduced number of cells.

BLV RNA expression greatly increased by factors present in FBS. A sizable increase in the number of BLV RNA-containing cells occurred when PBMCs were removed from blood and placed into culture, and FBS stimulated a greater increase than did autologous serum (Table 2). FBS is used in cultures as a source of growth factors and other regulators, so we investigated it as an activator in the culture medium.

We found that the number of cells containing BLV RNA when the cells were cultured with 1% FBS was less than the number observed in cultures with 10% FBS and was reduced even further in the absence of FBS (Fig. 3). Comparison of cells cultured in 10% FBS with those cultured without FBS showed that a large proportion of cells in 10% FBS contained small amounts of BLV RNA, suggesting that a population of cells was newly recruited to viral RNA synthesis by 10% FBS. Some cells transcribed BLV RNA without exposure to FBS; this expression may have been elicited by the release of platelet-derived factors during the process of purifying the cells from blood, as already discussed. These results, together with the apparent absence of an inhibitor in plasma, suggest that factors present in FBS stimulate infected PBMCs to make BLV RNA.

Increased BLV RNA expression in PBMCs caused by immune cell activators. Treatment of PBMCs from BLV-infected animals with phytohemagglutinin (PHA), a T-cell mitogen, causes increased expression of BLV in culture (3, 6, 14, 49). B-cell activators, such as LPS and PWM, reportedly do not stimulate synthesis of BLV virion proteins in PBMCs (6, 14). These results are puzzling, because BLV-induced tumors are thought to arise from B cells (4).

Since in situ hybridization allows examination of RNA in single cells, we tested immune cell activators for their early effects on BLV transcription before secondary events, such as cell survival and cell division, influenced the outcome. We treated PBMCs from infected sheep with LPS and found increased numbers of cells containing BLV RNA after 24 h of culture (data not shown). We then tested several T- and B-cell activators and mitogens for their effects on BLV expression in PBMCs obtained over a period of 8 months from four infected animals (Fig. 4). We found BLV RNA in a higher proportion of the PBMCs if they were treated for 6 to 8 h in culture with any one of the activators-mitogens than if they were cultured with 10% FBS alone. Treatment with either anti-immunoglobulin M antibodies or PWM increased the number of BLV RNA-containing cells without affecting the profile of BLV RNA content within cells in the expressing population. PHA, a T-cell mitogen, and LPS, an activator of B cells and monocytes, not only increased the numbers of cells containing BLV transcripts but elevated transcript levels within cells by as much as threefold. Treat-
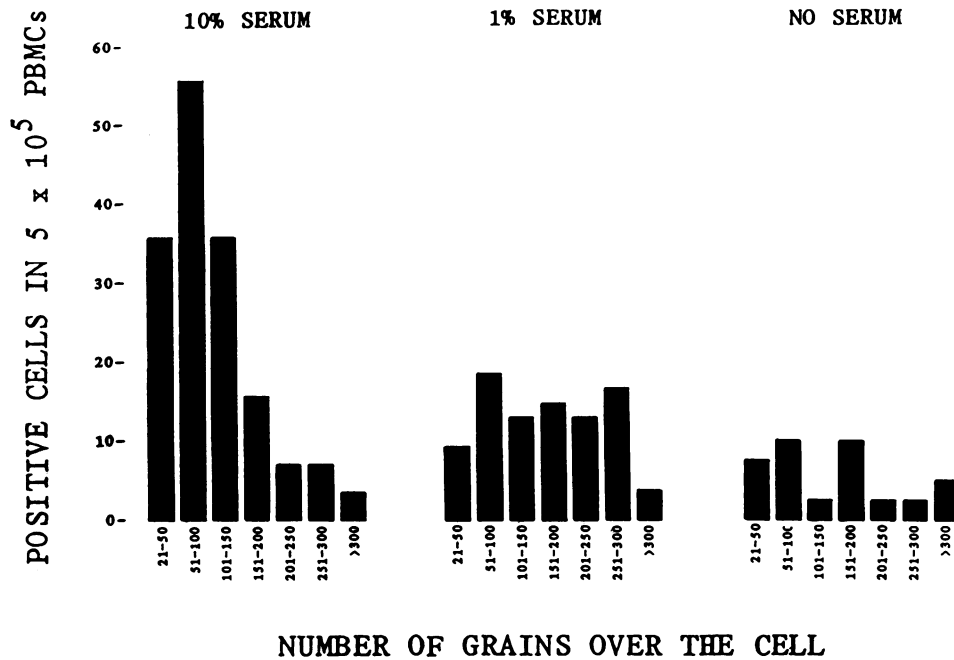
NUMBER OF GRAINS OVER THE CELL

FIG. 3. Dependence of BLV expression on serum factors. Isolated PBMCs were cultured for 6 h in medium without FBS, with 1% FBS, or with 10% FBS. Samples were analyzed after in situ hybridization as outlined in Materials and Methods. The grains over each positive cell in individual samples of 5×10^5 PBMCs were counted, and the cells were tallied for the number containing 21 to 50, 51 to 100, 101 to 150, 151 to 200, 201 to 250, 251 to 300, and over 300 grains. The data presented are from a representative experiment performed with cells taken from animal 380 at 24 months after it was experimentally infected with BLV. Cells fixed immediately after isolation from the blood contained no detectable BLV RNA in any of the 5×10^5 PBMCs.

ment with ConA, a T-cell mitogen, induced a high level of viral transcription in a small population of cells.

Figure 4 presents a composite of results from many experiments. With the exception of ConA, treatment with an activator or mitogen consistently increased the number of BLV RNA-containing cells in the population compared with control cells cultured in medium with 10% FBS. A more complex picture arose when the level of transcription in individual cells was examined. On some days on which blood was taken, LPS and PHA affected only the number of cells expressing viral RNA, not the level of transcription within individual cells. This occurred with all of the animals and was not unique to a particular experiment, since on two separate days the PBMCs of one animal were responsive and those of another were nonresponsive to mitogen-induced increases in BLV RNA within infected cells. Approximately one-third of our experiments were performed on days when PBMCs from a particular animal did not show this increase. Occasional failure of infected cells to increase virion production after stimulation by PHA has been previously documented (3, 49). On days when mitogens fail to increase the level of viral RNA in expressing cells, the immune system of the animal may have already been stimulated for maximal transcriptional activity of certain genes.

In summary, the number of BLV transcripts present in a mixed population of mononuclear cells can be increased over that obtained by culture in 10% FBS when the cells are exposed to a variety of immune cell activators or mitogens.

DISCUSSION

Certain retroviruses that infect cells of the immune system become latent after the initial infection and may not cause disease for many years. For HIV, evidence is accumulating

to suggest that immune activation of the T-cell host increases virus expression and in so doing may decrease the asymptomatic or latency period in vivo. HIV expression in cells in vitro is increased by certain T-cell activators (23, 34, 55) via increased HIV long terminal repeat (LTR) activity (48, 50). Clinical studies suggest that immune activation by exposure to other infectious agents may accelerate the development of the acquired immunodeficiency syndrome in HIV-infected individuals (38).

We report here a study of activation of BLV, a retrovirus that infects and induces malignancies of B lymphocytes. Activation of B cells to undergo proliferation and differentiation to antibody-producing cells can occur by distinct pathways that involve different regulatory signals (reviewed in references 5 and 10). We tested five different agents known to stimulate immune cells and found that all positively affect BLV transcription when added to a mixture of B cells, T cells, and monocytes isolated from BLV-infected animals. The mitogens could directly stimulate transcription within host cells of one or several lineages, or they could act through soluble mediators released locally within the mixed cultures. Factors in FBS also elicited BLV transcription in this cell population. Because these cells were examined for BLV transcripts within 8 h of treatment, and progression of B cells from quiescence into the G1 phase of the cell cycle probably requires 16 to 24 h (reviewed in reference 17), our data suggest that the cellular factors mediating transcription from the BLV LTR are positively regulated at a very early step in B-cell activation.

If BLV is transcribed only when infected cells are activated, as our results suggest, it is not surprising that BLV transcription in PBMCs would be extremely rare in vivo. Activation of B cells takes place largely in the microenvi-
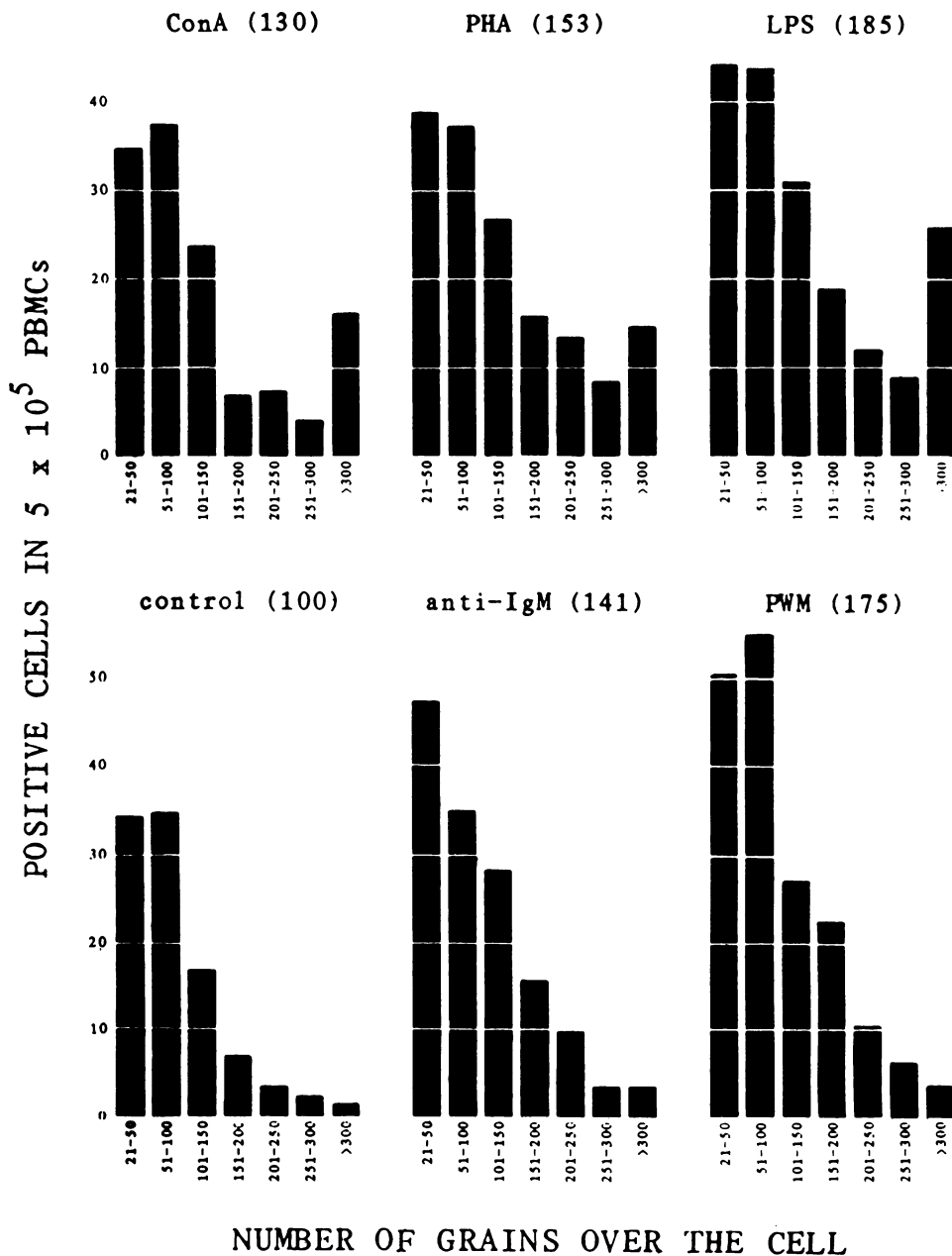
NUMBER OF GRAINS OVER THE CELL

FIG. 4. Mitogen stimulation of BLV expression. Blood samples for these experiments were collected from four sheep that had been infected with BLV for about 2 years and were in latency. The samples chosen for further analysis were those that yielded 30 to 200 BLV RNA-containing cells in 5 × 10⁵ PBMCs when cultured with no mitogen for 8 h in IMDM containing 10% FBS. PBMCs were cultured for approximately 8 h with IMDM containing 10% FBS (control) or with IMDM containing 10% FBS and supplemented with the indicated mitogen at the following concentrations: 50 μg of LPS per ml, 5 μg of PHA per ml, 20 μg of PWM per ml, 1.5 μg of ConA per ml, 20 μg of anti-immunoglobulin M per ml. After in situ hybridization, the data were analyzed as for Fig. 3. Each panel is a composite of results from experiments with different animals. The total numbers of experiments for the treatments represented were as follows: control, 6; anti-immunoglobulin M, 5; PWM, 3; ConA, 5; PHA, 4; LPS, 6. The number of BLV RNA-containing cells found in a sample of 5 × 10⁵ cells treated with each agent was calculated as a percentage of the control and is given in parentheses.

ronment of the spleen and lymph nodes. Hence, it is likely that in these loci of concentrated B cells, virus production and infection of new host cells occur. The cells that we activated to transcribe BLV in this study may have become infected in the spleen or in a lymph node during an immune response and may have been released later into the circulation. PBMCs from experimentally infected animals thus serve as an easily accessible source of material with which to study activation of a latent BLV provirus.

Our results showing that a variety of mitogens can activate transcription of a latent BLV provirus may appear to conflict with previous reports that PHA, but not PWM (6) or LPS (6, 14), increases BLV expression. However, several elements of our study differ. (i) We used a very sensitive technique to detect BLV transcripts that were produced at the earliest times after the rare, infected cells from animals in latency began to synthesize viral RNA. Augmentation of BLV transcription somewhat later (20 to 24 h) after exposure of

cells to PHA has been demonstrated by hybridization of RNA on dot blots (6), which requires that a large proportion of the cell population express viral RNA for detection. (ii) We have described stimuli that can activate transcription of BLV but have not asked what stimuli are necessary to obtain synthesis of virion proteins and production of viral particles. This distinction is important, because synthesis of nonstructural viral regulatory proteins that may affect host cell genes and tumorigenesis (19, 26, 33) could occur in the absence of further progression of the viral life cycle.

The increase in BLV RNA that we found in blood cells placed in culture appeared to be biphasic, with a slow rate of RNA synthesis followed by rapid enhancement of transcription. Although the viral transactivator proteins are necessary for efficient transcription from the viral promoter (7, 40), transcription of viral genes must begin in the absence of these proteins, since synthesis of the transactivator protein itself requires transcription of the viral genome. Cellular transcription factors induced by activation of lymphocytes may initiate a low level of transcription from viral promoters and so lead to synthesis of small amounts of the viral transactivator, which could then amplify transcription. The biphasic increase in BLV RNA that we observed may reflect such a sequence of events.

Transcriptional regulatory elements are found in the proviral LTR. DNase I protection assays suggest that the HTLV-I LTR is recognized not by viral proteins but by multiple host cell factors (1, 37). Interestingly, the BLV LTR sequence (8, 42) contains six bases, CTTTCC, at positions −87 to −82 that are part of the kappa light-chain enhancer (46) and are located in exactly the same position relative to the cap site as in the HIV LTR. Activation of T cells greatly increases factor binding to this kappa light-chain enhancer sequence with a concomitant increase in HIV expression (36).

NF-KB is a nuclear protein that binds the kappa light-chain enhancer (46) and is activated posttranslationally in pre-B cells treated with LPS or phorbol ester (47). An NF-KB-like factor responsive to B-cell activation may mediate initial, low-level BLV transcription. Higher levels of transcription could then be induced once the viral transactivator has been synthesized. The HTLV-I transactivator protein has recently been shown to induce factor binding to a kappa light-chainlike enhancer located upstream of the interleukin 2 receptor gene (32). Thus, cellular factors, such as NF-KB, may be involved not only as the initial activators of viral transcription but also as targets whose activities are modified by the viral transactivator protein. Although the mechanisms are not known, it seems likely that expression of a latent immune system retrovirus, whether it be BLV, HTLV, or HIV, depends on participation of host cell factors induced or modified during an immune response. If so, immune responses to unrelated antigens could facilitate spread of the virus and shorten the asymptomatic or latency stage of the disease.

ACKNOWLEDGMENTS

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