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Phytohemagglutinin Activation of the Transcription of the Bovine Leukemia Virus Genome Requires De Novo Protein Synthesis

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Addition of supramitogenic doses of phytohemagglutinin (PHA) to short-term cultures of neoplastic or nonneoplastic lymphocytes infected with bovine leukemia virus increased the synthesis of the major core virion antigen (p25) by 5- to 10-fold. Such stimulation was not due to the mitogenic effect of PHA or to a generalized increase in cellular RNA or protein synthesis but rather to enhanced transcription of the viral genome by a PHA-induced protein.

Infection of cattle with bovine leukemia virus (BLV), the etiological agent of the adult form of bovine lymphosarcoma (7), is usually persistent. However, the disease occurs in only a few infected animals. In naturally infected cattle, BLV has been detected only in B lymphocytes (19, 24). We have shown that in vivo the BLV-infected lymphocytes, regardless of whether they are neoplastic, usually harbor the viral genome in a repressed state (2, 8, 29) and that the repression occurs at the transcriptional level (8, 13). However, the BLV genome becomes rapidly derepressed after short-term cultivation of the cells (2, 8, 12, 13, 29). Other workers have also failed to detect viral RNA in noncultured neoplastic and nonneoplastic bovine lymphocytes (20, 21). We have shown that the transcriptional repression of the BLV genome in vivo is mediated by a nonimmunoglobulin protein termed the plasma BLV-blocking factor. This factor has been found only in the plasma, not in the serum, of BLV-infected cattle. It is not an antibody or an interferon molecule and does not block the expression of Rauscher murine leukemia virus or feline leukemia virus (12, 13).

Human T-cell leukemia virus (HTLV) like BLV, but unlike other mammalian C-type leukemia viruses, has been detected only after the infected cells were cultured in vitro (10, 26). Furthermore, it has been reported that the expression of the HTLV genome is blocked by a factor that, like the plasma BLV-blocking factor, is present in the plasma, but not in the serum, of an HTLV-infected patient (15).

Phytohemagglutinin (PHA) enhances the synthesis of virus particles (29) and viral antigens (2) in short-term cultures of BLV-infected lymphocytes. However, no information has been reported on the possible mechanism by which PHA, a T-cell mitogen, stimulates the expression of a B-tropic virus such as BLV. PHA has also been shown to induce the replication of herpes simplex (22) and mumps (5) viruses and to enhance the production of vesicular stomatitis virus (6), measles virus (17), and poliovirus (34) in human leukocyte cultures. For vesicular stomatitis virus, the enhancing effect has been attributed to increased lymphocyte metabolism resulting from the mitogenic activity of PHA (6). The increased replication of poliovirus in PHA-treated lymphocytes has been suggested to be due to more efficient adsorption and rapid eclipsing of the virus (34). The mechanism of the enhancing effect of PHA in the other viral systems is unknown.

The present study was undertaken to investigate the mechanism by which PHA enhances the expression of BLV in short-term cultures of BLV-infected bovine lymphocytes. The results reported here indicate that this mechanism does not involve the mitogenic effect of PHA but rather an enhanced transcription of the provirus by a PHA-induced protein.

In previous reports, immunofluorescence (2) and electron microscopy (29) were primarily used to show the stimulating effect of PHA on the synthesis of BLV antigen and BLV particles, respectively, in short-term lymphocyte cultures. To quantitate the PHA-induced stimulation of BLV expression in these cultures, we used the competitive radioimmunoassay (11) to measure the synthesis of the major internal virion protein p25. The addition of PHA to the medium increased the synthesis of BLV p25 by eightfold in cultures of nonneoplastic lymphocytes (Table 1). PHA-induced stimulation of BLV antigen synthesis was dose dependent. Optimum stimulation, which ranged from 5- to 10-fold, occurred at a concentration of 1.5 μg of purified PHA per ml of medium.

Significant PHA-induced stimulation of BLV p25 synthesis was detected as early as 6 h and reached maximum after 20 to 24 h of in vitro cultivation (Fig. 1). PHA-induced stimulation of BLV expression was observed in short-term lymphocyte cultures from all of the 13 BLV-infected cattle examined.

PHA also markedly stimulated the synthesis of BLV p25 in a B-lymphocyte subpopulation, which contained only 5 to 7% E rosette-forming cells, as well as in short-term cultures of cells from a BLV-induced tumor. It has been reported that most of the lymphocytes in BLV-induced tumors are B cells (30).

The fact that PHA stimulates BLV expression in lymphocyte cultures containing only a few T cells argues against the possibility that such stimulation is mediated by lymphokines secreted by PHA-stimulated T cells. Also arguing against this possibility is the fact that the stimulatory activity of PHA on BLV expression is seen as early as 6 h after cultivation. Detectable levels of lymphokines are, in general, produced by PHA-treated T cells only after 24 h of cultivation (16).

PHA, at the optimum dose for stimulation of BLV expression, did not affect the incorporation of [^{3}H]thymidine in 24-h cultures of BLV-infected lymphocytes (Fig. 2A). The optimal mitogenic dose of PHA for bovine lymphocytes, which is fourfold lower than that required for the optimal stimulation of BLV expression, also had no effect on

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TABLE 1. Effect of PHA on the expression of BLV p25 antigen in short-term cultures of BLV-infected nonneoplastic and neoplastic lymphoid cells

Cells cultured[a]	PHA	BLV p25 synthesized[b] (ng/ml of cell extract)	Stimulation
Unfractionated nonneoplastic PBLs[c]	−	38	
	+	300	eightfold
E-RFC-depleted nonneoplastic PBLs[d]	−	60	
	+	262	fivefold
BLV-induced tumor cells[e]	−	87	
	+	472	sixfold

[a] Cells were cultured at 37°C for 20 to 24 h at an initial density of 3×10^6 cells per ml in 10 ml of minimal essential medium supplemented with 20% heat-inactivated fetal bovine serum in the absence or presence of electrophoretically pure PHA (PHA-L, Vector Laboratories) at a final concentration of 1.5 μg/ml.

[b] As determined by the competitive radioimmunoassay for BLV p25 (11).

[c] Nonneoplastic peripheral blood lymphocytes (PBLs) were isolated from the heparinized blood of BLV-infected cows by Ficoll-Hypaque gradient centrifugation (33).

[d] PBLs were incubated with 2-aminoethyl isothiouronium bromide hydrobromide-treated sheep erythrocytes to form E rosettes. B-cell-enriched mononuclear leukocyte fraction was derived by depleting the PBLs of E rosette-forming cells (E-RFC) (25).

[e] A suspension of single cells from a BLV-induced tumor was prepared by perfusion with medium. Cells were washed twice with minimal essential medium containing 5% fetal bovine serum and then cultured.

[^{3}H]thymidine incorporation during the first 24 h of culture. Furthermore, mitomycin C, which is a potent inhibitor of DNA synthesis in cells of various origins, including bovine lymphocytes, did not have any detectable effect on PHA-induced stimulation of BLV p25 synthesis or on recovery of viable cells in the lymphocyte cultures (data not shown). Thus, it is apparent that the mitogenic activity of PHA is not responsible for its stimulatory effect on the expression of BLV. Other T- and B-cell mitogens, such as concanavalin A, pokeweed mitogen, and lipopolysaccharide, tested at various concentrations, had no effect on the synthesis of BLV p25 in short-term lymphocyte cultures (data not shown). Driscoll et al. (4) have also reported that concanavalin A has no effect on the amount of BLV antigen produced in lymphocyte cultures of most cattle.

Higher levels of p25 in the PHA-treated lymphocyte cultures may be due to an increased susceptibility of lymphocytes to virus infection. However, this is unlikely because, as shown above, mitomycin C, which blocks retrovirus multiplication by inhibiting cellular DNA synthesis (1, 31), does not interfere with the PHA-induced stimulation of BLV expression in lymphocyte cultures.

PHA at the optimum dose for stimulation of BLV expression had no effect on the incorporation of [^{3}H]uridine (Fig. 2B) or [^{3}H]leucine (Fig. 2C) in BLV-infected lymphocytes cultured for 24 h. These data indicated that the enhancing effect of PHA on BLV expression was not a consequence of a generalized increase in total cellular metabolism. Mitogenic doses of PHA, which were fourfold less than those required for the optimal stimulation of BLV expression, also failed to stimulate RNA and protein syntheses during the first 24 h in bovine lymphocyte culture (data not shown).

It was of interest to determine whether the PHA-induced stimulation of the synthesis of BLV p25 was due to an increase in the transcription of the viral genome. Cytoplasmic RNA isolated from cells cultured with or without PHA were analyzed for viral transcripts with the dot blot hybridization technique described by Gupta et al. (13). Figure 3 shows that the levels of BLV transcripts in lymphocytes cultured in the presence of PHA (row C) were six- to eightfold higher than in lymphocytes cultured without the mitogen (row A).

The possibility was considered that PHA-induced stimulation of BLV transcription occurs through an intermediary protein. In this study we used cycloheximide, a known inhibitor of protein synthesis in other systems. As determined by [^{3}H]leucine incorporation experiments, cycloheximide also inhibited protein synthesis in bovine lymphocytes (data not shown). To examine the effect of cycloheximide on the PHA-induced stimulation of BLV transcription, infected lymphocytes were cultured for 30 min in the presence of the drug (5 μg/ml). Subsequently, PHA was added to the culture as well as to an untreated parallel control culture. Cytoplasmic RNA was isolated from the cells after 20 h of cultivation and analyzed for BLV transcripts by the dot blot hybridization technique. The results showed (Fig. 3) that the treatment of the cells with cycloheximide abolished the PHA-induced stimulation of viral RNA synthesis (compare rows C and D). Cycloheximide did not have a deleterious effect on the synthesis of BLV transcripts in lymphocytes cultured without PHA (compare rows A and B).

From these results we concluded that the derepression of the BLV genome in lymphocytes on short-term cultivation does not require the synthesis of a new protein and that the PHA-induced stimulation of viral RNA synthesis in the cultures is mediated by a PHA-induced protein.

The increased transcription of the mouse mammary tumor virus genome by the glucocorticoid hormone has been shown to be due to an increase in the number of RNA polymerase II molecules transcribing the integrated viral DNA (9). It has been postulated that the binding of the

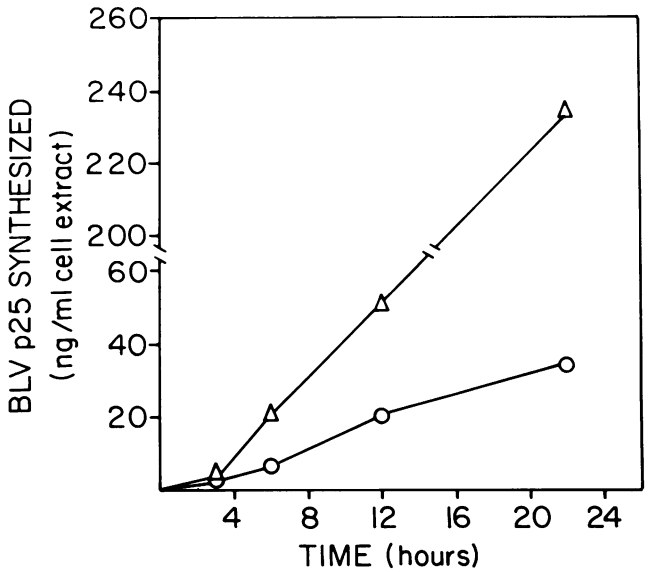

FIG. 1. Effect of PHA on the kinetics of BLV p25 synthesis in short-term lymphocyte cultures. Lymphocytes were cultured at a density of 3×10^6 cells per ml in minimal essential medium containing 20% fetal bovine serum in the presence (△) and absence (○) of PHA (1.5 μg/ml). At 3, 6, 12, and 22 h after cultivation, cells were harvested, washed, and examined for BLV p25 synthesis by the competitive radioimmunoassay (11).

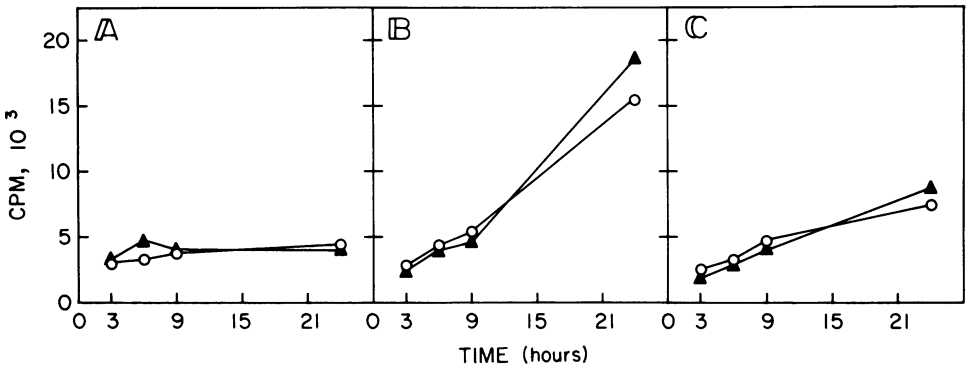

FIG. 2. Effect of PHA on the synthesis of cellular DNA, RNA, and protein in cultured BLV-infected PBLs. PBLs were cultured in the absence (○) or presence (▲) of PHA as described in Table 1, footnote *a*. [^{3}H]thymidine (panel A), [5, 6-^{3}H]uridine (panel B) or L-[4,5-^{3}H]leucine (panel C) were added to a final concentration of 5 μCi/ml of medium at zero time of culture. The incorporation of radioactivity into cellular DNA, RNA, and protein was determined by measuring trichloroacetic acid-precipitable radioactivity after 3, 6, 9, and 24 h of cultivation.

hormone-receptor complex to a specific DNA sequence results in a conformational change of the integrated viral genome such that the initiation of transcription can take place more frequently by providing entry to more RNA polymerase molecules (9). The protein induced by PHA in bovine lymphocytes may interact with certain DNA sequences, thereby increasing the polymerase loading on the proviral DNA.

The BLV genome, like the HTLV genome but unlike the genome of other known C-type viurses, has a region designated X, which is located between the envelope gene and the 3′ viral long terminal repeat (14, 27). Recent evidence suggests that a protein, Px, encoded by the X region of HTLV stimulates the transcriptional activity of the viral long terminal repeat (28). It would be of interest to determine whether PHA enhances the transcription of the BLV genome by increasing the synthesis of a similar BLV protein.

PHA-induced stimulation of viral transcription has not been demonstrated in any other retroviral systems. Studies on the mechanism of PHA-induced stimulation of BLV expression may help to gain an insight into the regulation of the expression of BLV, HTLV, and other as yet unidentified cryptic leukemia viruses of other species, and therefore contribute to their identification.

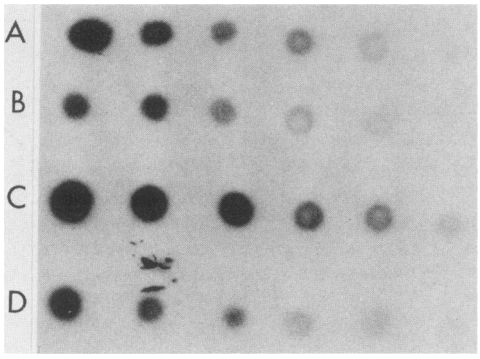

FIG. 3. Dot blot hybridization of viral RNA from BLV-infected lymphocytes cultured in the presence of PHA. The culture conditions were the same as those described in Table 1, footnote *a*, except that 500 ml of culture was used in each case. The cultured cells were harvested and washed three times with phosphate-buffered saline. Cytoplasmic RNA was extracted from the cultured cell pellet by the hot phenol procedure of Britten et al. (3). ^{32}P-labeled BLV DNA probe was prepared from cloned viral DNA (18) by the method of Maniatis et al. (23). The specific activity of the nick-translated probe was 8×10^8 cpm/μg. The dot blot hybridization assay was done by the method described by Thomas (32). Analysis of RNA isolated from BLV-infected lymphocytes cultured in medium only (row A), in medium with cycloheximide (5 μg/ml) (row B), in medium with PHA (row C), and in medium with cycloheximide (5 μ/ml) followed by the addition of PHA after 30 min (row D) was carried out in serial twofold dilutions.

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