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Bovine Leukemia Virus Replicates in Sheep B Lymphocytes under a T Cell Released Factor

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Abstract—*In order to determine which cell supports BLV replication in experimentally infected sheep, peripheral blood lymphocytes (PBL) were separated into purified B and T populations by a panning technique. Our data demonstrate that viral replication takes place only in B lymphocytes. However, PHA, a T cell mitogen, is necessary for BLV replication both in PBL and enriched surface immunoglobulin bearing cells, whereas B cell mitogens have no effect on viral replication. Altogether, these results suggest that BLV activation in enriched B lymphocytes is dependent on the presence of residual T cells, and occurs through a T cell interaction, probably mediated by a soluble factor. This possibility was confirmed by the fact that the conditioned medium from cultures of BLV-free sheep T lymphocytes greatly enhances viral production by infected B lymphocytes. Our data favor the hypothesis that BLV multiplication occurs through the regular activation mechanisms of the immune system.*

INTRODUCTION

THE bovine leukemia virus (BLV) is an exogenous retrovirus [1] that induces enzootic leukosis in cattle. All infected animals develop BLV specific antibodies, but only 30% develop a persistent lymphocytosis (PL) and 5% a lymphosarcoma [2]. In any case, viral production is not evidenced unless PBL or tumoral cells are put in short term culture. Moreover, only the B lymphocytes were demonstrated to support viral replication [3]. PHA generally greatly increases the rate of virus production, that may however occur in absence of this mitogen. Experimentally, BLV is infectious for sheep [4], which develop a lymphosarcoma with a high frequency in a relatively short time and exhibit a high titered antibody response against BLV antigens [5]. The present work aimed at studying which cell supports viral replication in infected sheep. In this species, we demonstrate that virus production takes place in the B lymphocytes [6], occurs only in the presence of PHA and involves the presence of T cells or of a T cell-released factor. As recent studies emphasize the common functional and structural features between BLV and the human lymphotropic retroviruses HTLV-I, II and III [7], the ovine system may therefore constitute a good experimental model for the virus-induced leukemias of the BLV-HTLV group.

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MATERIALS AND METHODS

Animals

Fifteen Prealpine 6-month-old sheep were inoculated intraperitoneally with 5.10^7 lymphocytes from a BLV-positive cow with PL and five animals were used as control. At the time of this study, all animals were 36 months old and the infected sheep had a peripheral leukocyte count from 7000 to 120,000 cells/mm^3, with a differential lymphocyte count from 60 to 90%, consisting mostly of surface immunoglobulin bearing cells (SIg+). The control contact sheep remained clinically and hematologically normal (5000–10,000 leukocytes/mm^3, with 55–65% lymphocytes) and negative for the presence of anti BLV antibodies.

Lymphocyte preparation

Venous blood was defibrinated on glass beads and the lymphocytes were isolated by the Ficoll technique after carbonyl iron incubation for monocyte depletion. After two washes in phosphate buffer serum (PBS), the cells were adjusted at 2.10^6/ml in RPMI 1640 medium with 10% heat inactivated fetal calf serum (FCS) and 16 mM hepes buffer.

Lymphocytes markers

(1) *Surface immunoglobulin (SIg).* B lymphocytes were characterized by the presence of SIg in a direct immunofluorescence (IF) test. The

peripheral blood lymphocytes (PBL) (1.10^6) were washed in PBS and suspended in 50 µl of a 1 : 30 dilution of a fluorescein conjugated rabbit anti-sheep immunoglobulin antiserum (Nordic). After a 30-min incubation at 4° C, the cells were washed three times with PBS in a refrigerated centrifuge and the preparation was examined by fluorescent microscopy. Percentage of SIg+ cells was established by counting at least 200 cells.

(2) *PNA-binding cells.* Cells (1.10^6) in 50 µl of PBS were reacted with 10 µl of a 1 mg/ml solution FITC-labelled PNA (Vectors lab.) for 30 min at 4° C. The cells were washed three times by centrifugation in the same medium and the differential count established by counting at least 200 cells.

Panning separation of B and T lymphocytes

The method was adapted by Wysocki and Sato [8]. Sterilin 100 × 15 mm polystirene bacteriological Petri dishes were precoated by overnight incubation at 4° C with 10 ml of 0.250 mg/ml immuno-absorption purified preparation of goat anti sheep heavy and light chains immunoglobulins (Biosys Laboratory, France). Before use, the plates were washed three times with PBS and received 10 ml of a 10.10^6 cells/ml lymphocyte suspension. After a 30-min incubation at room temperature, the non-adherent cells were resuspended by swirling and pipetted off. The plates were then washed 5 times with PBS to remove residual non-adherent cells. These non-attached cells were given a second cycle of incubation onto another precoated plate and similarly processed. Finally the adherent cells of all plates were recovered by vigorous pipetting with PBS. The non-adherent and adherent subpopulations were washed and adjusted at 2.10^6 cells/ml and cultivated in RPMI 1640 with 10% fetal calf serum. Phytohemagglutinin-P (PHA) (Difco), lipopolysaccharaide (LPS) from *S. typhosa* (Difco) or dextran sulfate 500 (DS-500), were added at various concentrations in the culture medium.

Conditioned medium (CM)

After panning separation of PBL from a BLV negative sheep, non-adherent T cells were harvested and cultivated in presence of 50 µg/ml of PHA. Two days later, the culture was spun down (2000 ***g***, 15 min) and the supernatant filtered and used as culture medium after dilution with an equal amount of RPMI 1640 with 10% FCS.

BLV-antigens detection

Aliquots of 50 µl of the cell suspension were cytocentrifuged, the slides air dried and acetone-fixed. BLV-antigens were detected by an indirect immuno-fluorescence (IF) test, using 50 µl of a mixture of eight monoclonal anti BLV gp51 antibodies [9] and an FITC-conjugated rabbit anti mouse antiserum. As control, BLV negative cells were tested in the same assay.

Mitogen-induced proliferation

One hundred microlitres of cells at 2.10^6/ml were cultured with 100 µl of a 50 µg/ml solution of either PHA or LPS, in 96-well flat bottom Falcon tissue culture plates for 48 hr, were pulsed for the last 18 hr of culture with 0.5 µCi methyl-H3-thymidine (specific activity = 5µCi/mmol. Amersham), were harvested onto filter papers (Dynatech cell harvester, Flow laboratories) and the radioactivity was counted in a bêta counter (bêta scintillation spectrophotometer, Intertechnique France). Triplicate cultures were processed as described. The results are expressed as the mean radioactivity in counts per minute (cpm) or as stimulation indices (SI = cpm in presence of mitogen/cpm in absence of mitogen).

RESULTS

In all infected animals, virus expression, revealed by IF with anti-BLV gp51 monoclonal antibodies, was evidenced after short term culture of peripheral blood lymphocytes (PBL) and in presence of a minimal dose of 50 µg/ml of PHA (Table 1). The LPS or the dextran sulfate 500 had no effect on virus production. BLV could be detected as soon as 6 hr after the onset of the culture with PHA, and was maximal between 24 and 48 hr. Under the same culture conditions, all control uninfected animals remained negative for the presence of BLV antigens. In order to determine which lymphocyte population supported the virus replication, ovine B and T lymphocytes were separated by panning on anti-ovine immunoglobulin coated plates. Such a procedure allowed a recovery of 95% pure surface immunoglobulin bearing cells (SIg+) in the adherent population (Table 2) whereas, fluorescein conjugated peanut agglutinin (PNA), a T cell marker [10], labelled very few cells in the same population. Inversely, the non-adherent cells were highly labelled by PNA and had a minimal contamination by SIg+ cells (0–4%). As no potent and specific monoclonal antibody against ovine T lymphocytes was available, the depletion of residual T lymphocytes in the SIg+ population was not possible. However under optimal culture conditions, i.e. in the presence of 50 µg/ml of PHA and 48 hr culture at 37° C and 5% CO_2, only the adherent SIg+/PNA- subpopulation expressed BLV antigens (Table 3). The non-adherent SIg−/PNA+ subset remained persistently negative. The same results were obtained for all 15 infected animals, thus dem-

Table 1. *BLV antigen detection in* 48 hr *lymphocyte cultures*

Sheep	Medium	PHA (μg/ml)					LPS (μg/ml)			Dextran (μg/ml)
		1	10	25	50	75	10	50	100	40
Infected										
1	–	–	–	–	+	+	–	–	–	–
2	–	–	–	–	+	+	–	–	–	–
3	–	–	–	–	+	+	–	–	–	–
Control										
4	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–

Table 2. *Percentage of* SIg+ *and* PNA+ *lymphocytes before and after panning separation*

Cells	Sheep 1		Sheep 2		Sheep 3	
	SIg	PNA	SIg	PNA	SIg	PNA
PBL	79	20	51	38	59	24
A cells	94	3	95	2	95	3
NA cells	2	85	4	86	0	79

PBL = peripheral blood lymphocytes, A = adherent, NA = non adherent cells.

Table 3. *Detection of BLV gp*51 *in peripheral blood lymphocytes (PBL) and in the adherent (A) and non-adherent (NA) subpopulations after panning*

	–	PHA 25μg/ml	PHA 50μg/ml	CM
PBL	–	–	++	++
A cells	–	–	+	+++
NA cells	–	–	–	–

Percentage of IF positive cells was comprised between 5 to 15% (+), 15 to 25% (++), or 25 to 40% (+++).

Table 4. *Mitogen-induced proliferation. Results are expressed in* cpm *and, between parenthesis, stimulation indices* (cpm *in presence of mitogen*/cpm *in absence of mitogen*)

Cells	—	PHA	LPS
PBL	3869	5870 (1.52)	9685 (2.50)
A cells	4236	5158 (1.21)	17973 (4.24)
NA cells	4489	26207 (5.84)	3920 (0.87)

onstrating that in the ovine as in the bovine system, only the B lymphocytes support the BLV replication. This assumption can be made even though the panning technique never ended up with a 100% pure SIg+ population. In fact, the possibility of an overgrowth of residual T cells (SIg−/PNA+) in the adherent population was very unlikely based upon the demonstration that the percentage of SIg+ cells remained unchanged after 48 hr culture (always > 95%) (data not shown), and that no cell proliferation was observed after a 48 hr PHA stimulation (Table 4). On the other hand, the non-adherent cells kept their whole proliferative potentialities by responding as expected to this mitogen. Similarly, the adherent cells showed a good proliferative activity after LPS stimulation but no virus expression was ever observed. It thus may be concluded that BLV expression is not merely linked to the host cell multiplication. It may appear controversial that BLV replication is observed in the ovine B lymphocytes only after a stimulation by PHA, a T cell mitogen. Such virus induction through a B cell polyclonal activation by PHA seems very unlikely, especially because polyclonal B cell activators such as LPS or Dextran-sulfate-500 had no such effect (Table 1). On the contrary, BLV production may be related to the persistence after panning of a minor subset of PHA-responsive T cells in the adherent population that would induce virus expression by the SIg+ cells. In fact, virus production was less abundant after PHA stimulation in the SIg+ cells as compared to the whole lymphocyte population (Table 3). Altogether, these results suggest that BLV activation in the ovine B lymphocytes occurs through a T cell interaction. The possibility for this phenomenon to be mediated by a soluble factor was substantiated by the discovery that the conditioned medium (CM) from cultures of BLV-free sheep T lymphocytes was able to promote the BLV expression in both infected PBL and B cells (Table 3). As the CM was diluted 1 : 1 with fresh culture medium, the dose of residual PHA was not sufficient to activate the BLV expression (Table 3). PBL exhibited the same level of virus production when cultured in presence of either PHA or CM, whereas purified B lymphocytes showed higher amounts of BLV

positive cells in presence of CM. It is noteworthy that in any case, the percentage of virus producing cells was never more than 40%, even in the enriched SIg+ population. It thus may be concluded that only a subset of the B lymphocyte population is able to express BLV antigens, even under a direct stimulation by the CM.

DISCUSSION

Our results demonstrate that the B lymphocytes support viral replication in BLV-infected sheep. This is corroborated by the increase in the number of B lymphocytes in the leukemic form of the disease (manuscript in preparation), confirming data from Takashima and Olson [11]. However viral expression in peripheral lymphocytes is dependent on the presence of PHA, a T cell mitogen. The B cell mitogen, LPS, may activate only a subpopulation of B cells. As BLV-positive cells in IF also constitute a portion of ovine B cells, the possibility remained that the LPS-activated lymphocytes were not the BLV-producing cells. The dextran sulfate 500, another polyclonal B cell activator, had similarly no effect on virus production. This contrasts with the results obtained in mice where LPS was demonstrated to induce the release of endogenous xenotropic type C RNA virus from Balb/c spleen cells [12]. Our results on purified lymphocyte populations favor the hypothesis that a T cell soluble factor released after PHA stimulation induces the BLV expression in ovine B lymphocytes. It would be extremely important to study the role of this postulated factor on 100% pure B cells, situation where no viral expression at all under PHA stimulation is expected. And in fact, we commonly observed a relative decrease of BLV positive cells in the PHA-stimulated enriched SIg+ cell population as compared to the whole blood lymphocytes. On the contrary, an increase in BLV+ cells was found after culture of SIg+ cells in presence of CM. These data confirm a recent report [13] in the bovine system, where PHA was demonstrated to enhance BLV replication independently of cell proliferation. However the role of an eventual lymphokine was ruled out by these authors on the basis of the small number of residual rosette forming cells (5–7%) in a purified B lymphocyte population, and the fact that BLV production occurred before detectable levels of lymphokines are generally detected. It remains however possible that such a factor, immediately produced after PHA stimulation, is rapidly used by the responsive population, before reaching a detectable level. It is interesting to note that for the induction of BLV expression in the bovine system, the optimum dose of PHA concentration was reported to be 1.5 μg/ml [13]. In the ovine system and using an IF test for BLV antigen detection, no virus expression was observed unless a 50 μg/ml concentration of PHA was used. Such an apparent discrepancy may be related either to the different species under study or/and to the different origin of PHA preparations.

After antigen or mitogen activation, T lymphocytes produce several factors, categorized as B cell stimulating factors (BCGF, BCDF) which have activities in B cell activation and proliferation [14]. Such lymphokines probably also exist in the ovine system, and may be responsible for the activation of ovine B lymphocytes and possibly also for BLV expression. BLV as HTLVs possesses an additional gene named pX, the product of which is found to amplify but not initiate the viral gene transcription [7, 15]. Such a mechanism, likely to exist in BLV infected ovine lymphocytes, would contribute to the enhancement of viral replication. The postulated cell factor released after PHA stimulation would be able to initiate the viral replication. The cellular mechanism involved in the induction of BLV expression in sheep B cells is not understood. It may involve the triggering of a cell gene activation program which may also act on the provirus or its enhancer. In this regard, it is noteworthy that the BLV enhancer showed the greatest homology with the mouse immunoglobulin heavy chain gene (Cu) enhancer, which functions in a B cell specific manner [16]. On the other hand, it was demonstrated that the activation signals to the T cells are transmitted via the T3 peptide associated with the T cell receptor [17]. PHA, that specifically binds to this peptide [18], triggers a polyclonal activation of resting T cells. So, we may propose that, *in vivo*, any antigen after fixation to its specific T cell receptor induces, by the same mechanism, an activation of the responsive T cell clones that start producing soluble(s) factor(s) active on the corresponding specific B-cell clones in presence of the corresponding specific antigen. If this B clone was infected with BLV, the viral replication could be triggered. Such a mechanism involving a limited number of specific clones, would give rise to a very low level of BLV production, undetectable by common tests, but would account for the *in vivo* presence of high levels of viral antibodies. Our results suggest that multiple rounds of antigenic stimulation *in vivo* resulting in infection with various microorganisms or exposure to different agents (castration of BLV infected sheep leads to tumoral development [2]) may promote BLV expression and spread. If the transacting factor involved in enhancing proviral replication is implicated in activation of cellular oncogenes, the immunological activation of the proviral genome may lead to the tumoral development.

Despite a different cell tropism for BLV and

HTLV (B cells for BLV and T cells for HTLV), the replication pattern of these viruses presents many similitudes. It is noteworthy that upstream from the IL2 gene and a segment of the HTLV III/LAV LTR. Thus it has been postulated that homologous regions are common sequences involved in recognition of immunologic activation signals. Zagury *et al.* [19] proposed that *in vivo*, antigenic stimulation of an infected T cell results in HTLV III/LAV release, T4+ cell death, spread of the virus and ultimately an immunodeficiency syndrome. The pathological mechanism in BLV infection differs from the latter by the fact that BLV activation leads not to the cell death but to cell transformation.

Thus, BLV and HTLV III/LAV seem to use for their own replication and spreading the regular activation circuitry of the immune system.

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