Characteristics of Lymphocytes Appearing in Persistent Lymphocytosis Induced Experimentally in Cattle by Bovine Leukemia Virus Infection

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ABSTRACT. Properties of peripheral blood lymphocytes (PBL) appearing during persistent lymphocytosis (PL) of cattle that was induced by inoculation of a large number of bovine leukemia virus (BLV)-infected lymphocytes were studied. The PBL possessed surface immunoglobulin at a high ratio and had high syncytium-forming activity. The PBL could not be distinguished from normal PBL cytogenetically. These PBL were shown to be polyclonal cells integrating BLV-proviral DNA randomly into their cellular DNA. These properties were similar to those of PBL appearing at the stage of PL of cattle naturally infected with BLV. The BLV-proviral DNA content of PBL and transcription of viral genes increased in parallel with development of PL and decreased along with a decline of PL indicating the possibility that gene expression and activation of BLV play an important role in induction and maintenance of PL. **KEY WORDS:** bovine leukemia virus, cytogenetics, molecular biology, persistent lymphocytosis, surface immunoglobulin.

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Some cattle develop persistent lymphocytosis (PL) during the course of natural infection with bovine leukemia virus (BLV) [2, 4]. Major cell population of peripheral blood lymphocytes (PBL) at the time of PL (PLL) have characteristics of B lymphocytes [22, 26, 29, 30], show cytogenetic properties indistinguishable from those of noninfected cattle [7], produce a large number of infective BLV [13, 28, 36] and consist of polyclonal cells integrating BLV proviral genome at random sites in their cellular DNA [19].

BLV genome encodes *trans*-activator genes, which are essential for the expression of viral genes [16], and activate the bacterial chloramphenicol acetyl transferase gene directed by BLV-long terminal repeat (LTR) [18], at the XBL region that is located between *env* gene and 3' LTR [31, 33, 34]. The product of corresponding (pX) genes of human T cell leukemia viruses (HTLVs) [12, 35] which have many similar-

ities to XBL genome activate IL-2 receptor and IL-2 gene expression which may give rise to T lymphocyte proliferation [6, 27]. Therefore, it can be postulated that expression of the XBL genes have a role in promoting the division of B lymphocytes and inducing PL.

Integration and expression of the BLV genome in PBL of cattle chronically infected with BLV and cattle affected with enzootic bovine leukosis (EBL) have been studied [19–21], but there is no report on the BLV genome and its expression during the course of development of PL.

We reported in a previous paper [23] that cattle showed an increase in number of PBL suddenly after a latent period following inoculation of a large number of PLL. In this paper, characteristics of the PLL and the relationship between the rise and fall of the degree of PL and those of the BLV genome and its expression in PLL are described.

MATERIALS AND METHODS

Cattle: The animals used were one calf (B69) which developed PL after inoculation of 6.4×10^8 tumorous cells in the blood of a cow with EBL, 7 cattle (B5, 6, 7, 8, 9, 64, 67) which developed PL after being inoculated with $2.4\text{--}240 \times 10^7$ PLL, 1 calf (B71) which failed to develop PL after inoculation of 200 ml of culture fluid of fetal lamb kidney cells persistently infected with BLV (FLK-BLV) [39], and 5 cattle (B1, 2, 3, 4, 23) which did not develop PL after inoculation of cell-free BLV were used. Details of these cattle were given in a previous paper [23].

Detection of surface immunoglobulin (sIg)-positive cells: PBL collected by gradient centrifugation were stained with FITC-labelled anti-bovine IgG (H+L) serum (Cappel Laboratories, U.S.A.) by a routine method. The ratio of sIg-positive cells was determined by alternate observations of the same field by a fluorescence system and a phase contrast system of a microscope.

Syncytium-induction assay: The assay was performed as described previously [24].

Karyotype analysis: Analysis of individual karyotypes and chromosomal abnormalities of peripheral blood lymphocytes were performed by routine methods [10]. Twenty well-spread metaphases from each animal were analyzed. Lymphocytes cultured for 48 hours in a conventional medium containing 5-bromo-2'-deoxyuridine ($10 \mu\text{g/ml}$) were stained and then examined by the fluorescent plus Giemsa (FPG) method for sister chromatid exchange (SCE) [17]. Twenty cells on average (16–24 cells) were analyzed for each animal.

Radiolabelled viral probes: The following DNA fragments were prepared from a full-length proviral clone [14]; 1.05 kb fragment containing BLV 5' LTR and a part of

the *gag* gene for a LTR-*gag* probe [16], 6.8 kb fragment containing the *gag*, *pol*, and *env* genes for a representative (Rep) probe and 0.72 kb fragment of the XBL region for an XBL probe [14, 15]. These fragments were labelled ($1\text{--}2 \times 10^8$ cpm/ μm) with ^{32}P -dCTP (New England Nuclear, U.S.A.) by the nick translation procedure [32].

DNA isolation and Southern blot hybridization: Cellular DNA was prepared from PBL and tumor cells by sodium dodecyl sulfate (SDS)–proteinase K treatment and phenol extraction.

Cellular DNA ($5 \mu\text{g}$) was digested with restriction endonuclease (*Eco*RI or *Pvu*II), electrophoresed in 0.8% agarose, and blotted onto a nitrocellulose filter (Schleicher & Schuell, West Germany) according to the procedure described by Southern [36]. The filters blotted with *Eco*RI and *Pvu*II digests were hybridized with the LTR-*gag* and Rep probes, respectively, at 42°C in a solution containing 50% formamide, $5 \times \text{SSC}$ ($1 \times \text{SSC}$: 0.15 M NaCl, 0.015 M Na citrate), 0.5% SDS, $1 \times$ Denhardt's solution ($1 \times$ Denhardt's: 0.02% each of polyvinylpyrrolidone, bovine serum albumin and Ficol 400), 0.01 M ethylenediaminetetraacetic acid and salmon sperm DNA ($100 \mu\text{g}$ per ml), and washed at 68°C in $0.2 \times \text{SSC}$ –0.1% SDS. The filters were exposed to Kodak XAR-5 films at 70°C in the presence of Du Pont intensifying screens.

RNA isolation and dot blot hybridization: Total RNA was prepared from PBL and FLK-BLV cells by guanidine thiocyanate/cesium chloride centrifugation [5]. RNA dot blot hybridization was carried out according to the procedures described by Thomas [38]. In brief, total RNAs from PBL ($5 \mu\text{g}$) and FLK-BLV ($0.008\text{--}5 \mu\text{g}$) were spotted onto the nitrocellulose filter saturated with $20 \times \text{SSC}$. The filter was hybridized with the XBL probe at 42°C in a solution of 50% formamide, $5 \times \text{SSC}$ –50 mM sodium phosphate (pH6.5), $1 \times$ Denhardt's solution and dena-

Fig. 1. Responses of calves after inoculation of various sources of BLV. B67, 69, and 71 were inoculated intravenously with 2×10^9 PLL, 6.4×10^8 EBL cells and 200 ml of cell-free culture fluid obtained from FLK-BLV cells, respectively. The two dotted lines represent Bendixen's key [4] for determination of lymphocytosis. Values above the upper line, values between the two lines and values below the lower line indicate lymphocytosis, suspected lymphocytosis and normal, respectively. ¹Detection of BLV genome in PBL. —~++: intensity of hybridized band shown in Fig. 3 was digitalized. ²Infectivity of PBL shown by the SIA. —~++: no syncytia, and 1–9, 10–50, 51–250 syncytia/ 1×10^5 PBL, respectively.

tured salmon sperm DNA (250 $\mu\text{g/ml}$) and washed at 50°C in $0.1 \times \text{SSC}$ –0.1% SDS.

RESULTS

Ratio of sIgG bearing cells: In 1 (B69) and 3 cattle (B40, 64, 67) which were inoculated with EBL cells and PLL, respectively, the ratio of sIg-bearing lymphocytes increased to 58 to 90% after development of PL from 18 to 40% before the inoculation. An increase in sIgG bearing cells was also observed in 3 cattle (B23, 68, 71) which failed to develop PL after inoculation of cell-free virus, but the ratio remained in a

range of 45 to 65%. Representative cases are shown in Fig. 1.

Syncytium-forming activity of PBL: Seven cattle with or without PL were tested for the activity. It was higher in cattle with PL than in those without PL (Table 1). PBL of 2 cattle (B2 and 4) showed low syncytium-forming activity at the non-PL stage but the activity increased after development of PL which was induced by inoculation of a large number of PLL. In contrast, the activity was reduced when one animal (B22) recovered from PL.

Karyotype analysis: Chromosomal characteristics of 5 cattle were examined to disclose whether or not the lymphocytes propagated in recipient cattle originated in the donor lymphocytes. Karyotypes of a donor and 4 recipient were investigated when they developed marked PL. As shown in Table 2, PBL of the male donor had a characteristic karyotype of 59, XY; t (Robertsonian translocation 1/29), while PBL of 2 male recipients showed no such translocation and those of 2 female recipients showed female karyotype, XX.

PBL obtained from 6 cattle with PL and 5 cattle without PL were investigated cytogenetically (Table 3). There was no difference in incidence of numerical and structural chromosomal abnormalities between the 2 groups. Structural chromosomal abnormalities consisted of chromatid gaps (60.5%), chromatid breaks (34.9%) and fragments (4.7%). There was also no difference in frequency of SCE between the 2 groups. The rates of incidence of these chromosomal changes were in the normal range found in healthy cattle [10].

Analysis of viral genome in PBL: Donor and recipient PBL were examined for integration of BLV provirus to disclose the origin of the increased PBL in the recipient by Southern blotting analysis using the labelled LTR-gag probe. As shown in Fig. 2, PBL (circulating tumor cells) of a donor

Table 1. Relationship between the number of PBL and their syncytium-forming activity

Animal No.	dpi ^{a)}	PL	Nos. of PBL ($\times 10^3/\mu l$)	Syncytium-forming activity ^{b)}
B1	412	—	6	+
B3	412	—	6	+
B5	402	+	19	++++
B7	475	+	23	++++
B2	412	—	7	+
	479	+	16	++++
B4	412	—	7	+
	732	+	15	++++
B22	99	+	16	++++
	402	—	6	+

a) Days post inoculation with BLV

b) + and ++++: 1–9 and 51–250 syncytia per 1×10^5 lymphocytes

Table 2. Karyotype analysis of inoculated (donor) lymphocytes and increased (recipient) lymphocytes

Donor		Recipient	
Animal No. (Sex)	Karyotype	Animal No. (Sex)	Karyotype
B9 (male)	59, XY; t ^{a)}	B5 (male)	60, XY
		B6 (male)	60, XY
		B21 (female)	60, XX
		B22 (female)	59, XX; t

a) Heterozygous Robertsonian 1/29 translocation

Table 3. Frequency of lymphocytes with cytogenetic abnormalities in cattle with or without persistent lymphocytosis after infection with BLV

No. of cattle	PL	Chromosomal abnormalities			Sister chromatid exchange (SCE)		
		No. of cells analyzed	Numerical abnormalities	Structural abnormalities	No. of cells analyzed	No. of SCE	No. of SCE per cell
6	+	120	2 (1.7) ^{a)}	19 (15.8)	117	802	6.85
5	—	100	4 (4.0)	18 (18.0)	100	743	7.4
Spontaneous incidence in normal cattle ^{b)}			(2.4–4.9) (3.5) ^{c)}	(8.0–20.0) (16.6)	5.9–8.8 (7.1)		

a) Numbers in parentheses are the percent positive

b) Data investigated by Hanada and Muramatsu [10]

c) Average

Fig. 2. Sites of integration of BLV proviruses in the genomes of PBL of a donor (B28: a cow affected with EBL) and a recipient (B69). Details of the recipient are shown in Fig. 1. Cellular DNA samples (5 μ g) were digested with *Eco*RI and examined with a labelled LTR-*gag* probe.

cow (B28) with EBL contained 2 copies of the BLV genome corresponding to four bands, but randomly integrated BLV genome was found in PBL of a recipient (B69) by mean of smear band. These results indicate that PBL of recipient consist of newly infected polyclonal cells.

DNAs from PBL collected sequentially after inoculation from 3 calves were digested with restriction endonucleases and subjected to Southern blotting analysis. DNA samples digested with *Eco*RI were examined with the labelled BLV LTR-*gag* probe to detect the sites of integration of BLV provirus in the genomes of PBL. Those digested with *Pvu*II were examined with the labelled Rep BLV probe to estimate the number of integrated viral copies in the cells.

As shown in Fig. 3-A, viral DNAs were detected by the LTR-*gag* probe from 1 and 2 weeks post inoculation (wpi) to 5 wpi in 2 calves (B67 and 69) in accordance with development of PL. The number of viral

copies was reduced 5 wpi in B69 when the number of PBL recovered to nearly the normal range (Fig. 1). The viral genomes were found as smear during all stages of infection. In a calf, B71, that failed to develop PL the BLV genome could not be detected by the LTR-*gag* probe but was detected faintly 3 and 5 wpi by the Rep BLV probe.

Viral DNAs were also detected by the Rep BLV probe in parallel with the detection by the LTR-*gag* probe in PBL of the 2 calves (Fig. 3-B). The PBL were estimated to contain 1/3–1/2 a copy of the BLV genome, which may be complete, per diploid cell, when compared with the intensity of hybridization with BLV genomes which were obtained from an EBL cell line containing 1 BLV genome per diploid cell [15].

Transcription of the XBL region in PBL was investigated by RNA dot blot analysis. As shown in Fig. 4, the specific RNA derived from the XBL region was detected from 1 and 2 wpi in the 2 calves. The quantity of the RNA increased and decreased in parallel with the number of PBL. Also, sequential changes in the quantity coincided with those of proviral DNA in PBL. The quantity of the RNA was from 1/125 to 1/25 of that in FLK-BLV cells. The RNA was not detected in a calf (B71) which failed to develop PL.

DISCUSSION

A rapid increase in PBL was observed after a latent period following inoculation of cattle with a large number of PLL [23]. Karyotype analysis and molecular biological analysis of donor and recipient PBL showed that the increased PBL were derived from the recipient, not from the donor.

The majority of the PBL bore sIg. These PBL produced a large number of infective BLV. The PBL did not show the anomalous karyotype changes that are observed in

Fig. 3. Appearances of BLV proviruses in PBL after inoculation of BLV. (A): Cellular DNA samples (5 μ g) were digested with *Eco*RI and examined with a labelled LTR-*gag* probe. (B): The samples were digested with *Pvu*II and examined with a labelled Rep BLV probe. The sample in the farthest right lane is a control containing 1 BLV copy per diploid cell [15]. Details of the cattle are shown in Fig. 1.

cattle affected with EBL [7, 11], and their karyotypes were indistinguishable from the normal chromosomal pattern [10]. Furthermore, the increased PBL were shown to be polyclonal cells integrating BLV proviral genome at random sites. These characteristics of PLL were identical with those observed in PLL of cattle naturally infected with BLV [7, 13, 19, 22, 26, 28–30, 37].

A larger amount of BLV genome and higher infectivity were detected in PBL of cattle with PL than in those from cattle without PL. Also, the rise and fall in the number of PBL coincided with increase and decreases in the BLV genome in the PBL and infectivity of the cells. On the assumption that a diploid cell contains one proviral genome, it was estimated that about 30 to

50% of the PBL in PL cattle contained the BLV genome. In contrast, only a small percentage of the PBL of non-PL cattle contained the BLV genome. Furthermore, although transcription of the BLV genome was detected in accordance with the increase in the BLV genome in PBL of PL cattle. These findings indicate the possibility that genome expression and replication of BLV play an important role in induction and maintenance of PL.

In this study, transcription of XBL genes, which are essential for the expression of viral genes directed by BLV-LTR [16, 18] and suspected of enhancing the growth of lymphocytes as shown in the case of HTLVs [6, 27], was found in accordance with the rise and fall in the number of PBL and in

Fig. 4. (A): Appearance of XBL-region specific RNA in PBL of cattle infected with BLV. The RNA was detected by RNA (5 µg/dot) dot blot hybridization using a labelled BLV-specific DNA probe. Details of the cattle are shown in Fig. 1. (B): Positive control for the specific RNA. Cellular RNA extracted from FLK-BLV was diluted serially and tested.

parallel with the increase and decrease in BLV provirus in the PBL. Therefore, the expression of XBL genes may have a close relationship with development of PL, although it is not clear that the XBL gene function directly activate the BLV infected B lymphocytes. Studies of the relationship between the rise and fall of PL and appearance of a plasma factor which inhibits transcription of the BLV genome [8, 9] may disclose the significance of the transcription of the XBL genes for the development of PL.

It has been shown that transcription of the BLV genome is not detected in cattle naturally infected with the virus [20], although some expression of the genome was suggested by an increase in specific antibodies during the course of development of lymphosarcoma [25]. However, we

found the transcription of XBL region of bovine leukemia provirus in cattle at an early stage of infection in this study and also revealed expression of the provirus in the early stage as a transient viremia in a previous study [24]. Although the difference in results obtained in these studies seems to be derived from the difference in the stage of infection, a longer sequential investigation of cattle with PL will be useful to elucidate a state of BLV-genome expression during a stage of chronic infection.

Development of anti-BLV antibody which inhibits the maturation of BLV *in vivo* and *in vitro* [1, 3] did not suppress the transcription of XBL genes. This finding suggests the continuous expression of the genes during the stage of PL until development of the plasma factor [8, 9].

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要 約

牛白血病ウイルス接種により実験的に牛に誘起された持続性リンパ球症時のリンパ球の性状：甲野雄次・糸原重美・村松 晋¹⁾・泉対 博・石野清之・関川賢二（家畜衛生試験場，¹⁾畜産試験場）——牛白血病ウイルス（BLV）感染リンパ球の大量接種により発生した持続性リンパ球症（PL）時の末梢血リンパ球（PBL）の性状を明らかにした。PBLは表面免疫グロブリンを持ち、高いシンチウム形成能を示し、細胞遺伝学的には正常リンパ球と区別できなかった。また細胞DNA中にBLVプロウイルスDNAをランダムに取込んだポリクローナルな細胞群であった。これらの性状はBLVに自然感染したPL牛のPBLの性状と同一であった。PBLのBLVプロウイルス含有量とウイルス遺伝子の転写はPLの発現とともに増加し、その減衰とともに減少し、BLV遺伝子の発現とウイルスの増殖がPLの成立と密接な関係を持つことを示唆した。