

## 牛白血病ウイルス接種により起こる牛の持続性リンパ球増多症とその継代

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# Development and Serial Passage of Persistent Lymphocytosis Associated with Bovine Leukemia Virus Infection in Cattle

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**ABSTRACT.** Two calves each were inoculated with  $1.5 \times 10^8$  or  $5 \times 10^9$  lymphocytes collected from each one cow which had persistent lymphocytosis (PL) and antibodies to bovine leukemia virus (BLV). A sudden increase in the number of peripheral blood lymphocytes (PBL) was observed 14 and 23 days, respectively, after inoculation and the maximum number reached 29,000 and 52,000/ $\mu$ l 72 and 57 days after inoculation. Although the degree of PL decreased gradually in these cattle, it continued until 14 and 44 months after inoculation when one animal was sacrificed and the other died of lymphosarcoma. The PL was passaged in cattle by inoculation of a large number of PBL obtained from cattle at the stage of PL (PLL). The degree of PL was severer in cattle inoculated with a larger number of PLL. PL was not caused by inoculation of PBL obtained from either BLV-infected non-PL cattle or cattle free of BLV. The PL was also caused by inoculation of PLL into BLV-infected non-PL cattle. On the other hand, it was not observed after inoculation of a large amount of cell-free virus obtained from short-term cultures of PLL. Antibodies to BLV developed earlier and to higher levels in cattle inoculated with PLL than in those inoculated with cell-free virus. These facts show that infection with BLV was established more effectively by PLL than by cell-free virus, the infection may occur by lymphocyte to lymphocyte interaction and the actual number of infected BLV may have an important role in development of PL.—**KEY WORDS:** bovine leukemia virus, lymphosarcoma, persistent lymphocytosis.

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Bovine leukemia virus (BLV) infection in cattle is characterized by two different proliferative responses of the host's B lymphocytes. One of them is the rare but fatal lymphosarcoma and the other is subclinical and benign proliferation of peripheral blood lymphocytes (PBL) [6, 11]. The latter, persistent lymphocytosis (PL), is also not a very frequent response in naturally [1, 13, 14, 39] and experimentally [34, 36, 41] infected cattle. PBL appearing during the stage of PL (PLL) is predominantly composed of cells having B-cell characteristics [29, 30, 35, 38] and cannot be distinguished morphologically [31, 45] and cytogenetically [18] from PBL of normal cattle although they produce a large number of BLV particles *in vitro* [22, 33, 43].

It has been considered that genetic factors

of the host may play an important role in the development of both PL and lymphosarcoma [1, 2, 12, 32] and that the factors controlling the development of PL and lymphosarcoma are independent [1, 13].

Although the mechanisms of development of PL and lymphosarcoma are quite obscure, the transacting transcriptional regulation hypothesis [42] proposed for transformation by human T-cell leukemia viruses (HTLVs) that have many similarities to BLV [7, 46] might open up a way of understanding tumorigenesis by BLV. It has been thought that transformation of lymphocytes by chronic transforming retroviruses may be a multi-step process. An analysis of the proliferative response of PBL may be helpful for understanding the step of malignant cell proliferation induced by BLV

infection.

Development of PL in cattle infected experimentally with various inoculums was reported before and after findings of the etiological agent of bovine leukosis, BLV. However, pathogenesis of PL could not be studied since it occurred after various long incubation periods and at a low rate [34, 41]. During etiological studies on BLV we found that cattle develop PL suddenly and consistently after inoculation of a large number of PLL.

In this paper we describe the conditions for development of PL in cattle. The properties of PLL will be described in a following paper [26].

#### MATERIALS AND METHODS

*Donor cattle for PBL used for the primary inoculations:* The following cattle kept in different regions were used as donors of PBL. 1) One cow (T324, Holstein) in a herd (about 120 head) in which there were many cases of PL but no case of enzootic bovine leukosis (EBL). The cow had 28,000 PBL per  $\mu\text{l}$  at the time of collection. 2) One cow (C69, Japanese Black) in a herd (about 90 head) in which several cases of EBL were reported. The number of PBL was 15,500/ $\mu\text{l}$ . 3) One cow (G2, Japanese Black) that had been kept in our institute and showed neither PL nor a temporary increase in number of PBL during 3 years. The number of PBL was 4,200/ $\mu\text{l}$  at the time of their collection. All these 3 of cattle were positive for BLV in the immunodiffusion (ID) test and the syncytium induction assay (SIA). 4) Two control cattle (B51 and B563, Japanese Black) that have been kept in our institute for several years and proved to be free of BLV infection by the ID test and the SIA.

*Inoculums:* PBL, BLV-infected cultured cells and cell-free BLV were used. Lymphocytes were collected by gradient centrifugation [5] of heparinized peripheral blood of

the cattle. Fetal lamb kidney cells which were persistently infected with BLV (FLK-BLV) [44] were obtained by trypsinization of the cell culture. These cells were suspended in appropriate volumes of Eagle's minimal essential medium and inoculated promptly into cattle. Culture fluids obtained from bovine embryonic thymus cells which were persistently infected with BLV (BET-BLV) and FLK-BLV cells and short-term culture [9, 33] fluids of PLL of one animal (B9) and tumor cells of a cow with EBL were used as for cell-free virus inoculum after removal of the cells by centrifugation. These culture fluids were shown to contain the core-protein (p24) antigen of BLV by the ID test after the concentration 100 times as shown in Table 2.

The number of cells inoculated and the amount of the inoculum are shown in the text and in Tables 1 and 2. Each substance was divided into three equal volumes and each of them was inoculated intravenously, intraperitoneally or subcutaneously into a calf.

*Animals:* Twenty-six cattle (17 Japanese Black and 9 Holstein), 1.5 to 18 months old, which were confirmed to be free of BLV by the ID test and SIA, were used. The ages at inoculation are shown in Tables 1 and 2. These cattle were kept individually in an isolated pen throughout the experimental periods.

*Hematological tests:* The tests were performed daily to once a week in the early stage of infection and once or twice a month during the late stage. An animal was regarded as having PL when the absolute lymphocyte counts fit the criteria for lymphocytosis [4] for at least 2 months.

*ID test:* The test for antibody against the glycoprotein antigen of BLV was as described previously [28].

*SIA:* The assay was conducted as described previously [27].

## RESULTS

*Primary inoculation with PBL obtained from cattle infected naturally with BLV:* Changes of the number of PBL in 3 cattle inoculated with PBL obtained from 3 different sources are shown in Fig. 1. In the calf (B9) inoculated with  $5 \times 10^9$  PBL collected from one (T324) of the cows with PL the number of PBL had decreased to  $2,000/\mu\text{l}$  5 days post inoculation (dpi), began to increase dramatically 23 dpi and then reached the maximum,  $52,000/\mu\text{l}$ , 57 dpi. The number decreased gradually thereafter and remained between  $15,000$  and  $20,000/\mu\text{l}$  for the 3 to 20 months post inoculation (mpi). The animal had  $9,000$  to  $15,000$  PBL per  $\mu\text{l}$

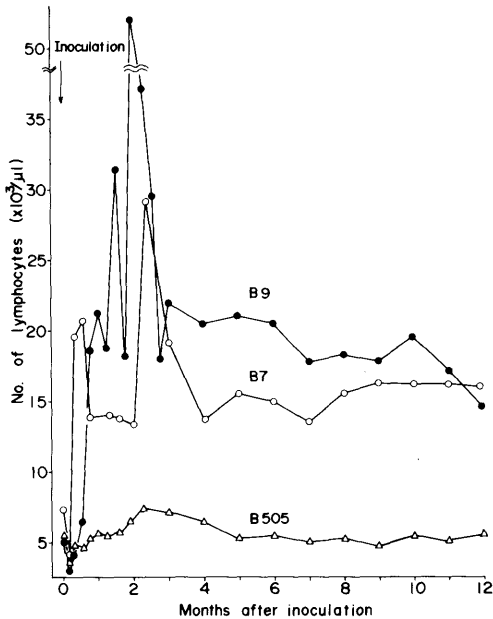


Fig. 1. Changes in number of PBL in cattle inoculated with PBL collected from 3 donors naturally infected with BLV. B7 and B9 were inoculated with  $1.5 \times 10^8$  and  $5 \times 10^9$  PBL collected from 2 donors with PL (T324 and C69), respectively. B505 was inoculated with  $1.5 \times 10^9$  PBL collected from a donor (G2) without PL. The number of PBL of each test was plotted until 2 mpi and thereafter the average number of 2 to 4 tests per month was shown.

until 44 mpi when it died of emaciation with histopathological changes of lymphosarcoma [21]. In the calf (B7) inoculated with  $1.5 \times 10^8$  PBL from another cow (C69) with PL, the changes in number of PBL were essentially similar to those of B9.

The calf (B505) inoculated with  $1.5 \times 10^9$  PBL from the cow (G2) without PL showed a very mild increase in number of PBL which was below the level for PL for a few months after inoculation and then the number returned to the preinoculation level. The maximum number of PBL was  $7,500/\mu\text{l}$ .

On the other hand, 3 calves inoculated with  $2-3 \times 10^9$  PBL obtained from 2 control cattle showed no changes in the number of lymphocytes during observation periods of 6 months, except for a decrease in the number observed within 7 dpi.

*Serial inoculation of PLL:* The results of serial passages of PLL starting calf B9 are summarized in Table 1. Pairs of calves (B6 and B22, and B5 and B21) were inoculated respectively with  $2.4 \times 10^7$  and  $2.4 \times 10^9$  PBL obtained 57 dpi from B9, when the animal showed the highest number of PBL. Response of the calves was essentially the same as that observed at the primary inoculation test (Fig. 2). All calves showed a rapid increase in number of PBL between 14 and 28 dpi and the highest numbers in each animal,  $14,000$ ,  $21,000$ ,  $24,000$  and  $28,000/\mu\text{l}$ , were observed 35 to 56 dpi. The persistently elevated PBL number was maintained in 3 cattle until 20 to 24 mpi when the animals were sacrificed. In 1 animal, B22, PL was observed until 5 mpi and then the number of PBL returned to the normal level. The increase in number of PBL was more marked in the 2 cattle inoculated with  $2 \times 10^9$  cells than in the 2 inoculated with  $2 \times 10^7$  cells.

Furthermore,  $1 \times 10^9$  PBL obtained from B9 on 823 and 968 dpi, when the donor had apparent PL and inapparent PL, respectively (Table 1), were inoculated into calves B32

and B38, respectively. Calf B32 showed apparent PL and calf B38 showed only an intermittent increase in PBL.

Serial passage of PLL was continued with PLL of 2 donors (B5 and B32) as shown in Table 1. PL was found serially in all cattle through the 5th generation. However, the time of onset, duration and extent of PL differed with the individual animal.

*Inoculation of cell-free BLV or BLV-infected nonlymphoid cells:* Cell-free materials obtained from short-term cultures of PLL and tumor cells and culture fluids of BET-BLV cells and FLK-BLV cells were inoculated into cattle as shown in Table 2. FLK-BLV cells were also inoculated into a calf. All of them failed to develop PL during observation periods of 4 to 19 months, although they developed antibodies to

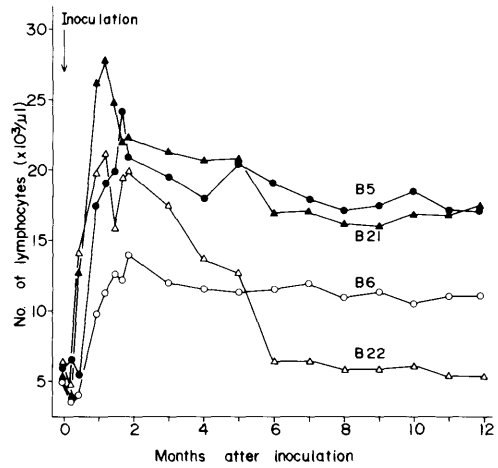


Fig. 2. Changes in number of PBL in cattle inoculated with PBL collected from donor B9. B5 and B21 were inoculated with  $2.4 \times 10^9$  PBL and B6 and B22 with  $2.4 \times 10^7$  PBL. The number of PBL of each test was plotted until 2 mpi and thereafter the average number of 2 to 4 tests per month was shown.

Table 1. Induction of PL in cattle by serial passage of a large number of PLL

Passage number in cattle					Age at inoculation (months)	No. of lymphocytes inoculated	First increase in lymphocytes	Max. No. of lymphocytes/ $\mu$ l of blood	Duration of PL (months)
1	2	3	4	5					
B9 <sup>a)</sup>					18	$5.0 \times 10^9$	23 <sup>b)</sup>	51,600	43
	B5				17	$2.4 \times 10^9$	28	26,200	20
		249/19.5							
			B501		6	$3.9 \times 10^8$	34	15,000	6
			B502		6	$3.9 \times 10^6$	20	17,000	6
	B6				18	$2.4 \times 10^7$	28	17,100	21
	B21				8	$2.4 \times 10^9$	14	28,000	23
	B22				8	$2.4 \times 10^7$	14	22,000	4
	B32				7	$1.0 \times 10^9$	11	16,500	4
		145/10.7							
			B40		6	$1.0 \times 10^9$	14	14,600	16
				603/10.1					
					4	$1.5 \times 10^9$	20	17,800	4
				43/15.2					
					1.5	$2.0 \times 10^9$	9	22,500	4
	B38				6	$1.0 \times 10^9$	28	12,500	*d)

a) Animal No.

b) Days after inoculation

c) PBL obtained 57 dpi (shown as the numerator) from animal B9 were inoculated into calves (B5, B6, B21, B22) when the number of PBL of the donor was  $52.0 \times 10^3/\mu$ l (shown as the denominator).

d) This animal showed an intermittent increase in the number of lymphocytes beginning 28 dpi, but did not have PL.

Table 2. Development of PL in cattle inoculated with cell-free BLV or nonlymphoid cells persistently infected with BLV

Animal No.	Age at inoculation (months)	Inoculum and dose (ml)	Antigen titer of inoculum <sup>a)</sup>	Development of PL	Observation period (months)
B1	14	BET-BLV <sup>b)</sup> cult. fluid, 300 ml <sup>c)</sup>	2	—	16
B2	14	ditto	2	—	16
B3	13	EBL cult. fluid <sup>d)</sup> , 300 ml <sup>e)</sup>	2	—	19
B4	14	ditto	2	—	19
B8	16	B9-PLL cult. fluid <sup>e)</sup> , 100 ml <sup>e)</sup>	2	—	4
B23	8	ditto	2	—	4
B68	1.5	FLK-BLV <sup>f)</sup> cult. fluid, 200 ml <sup>e)</sup>	160	—	5
B71	2	ditto	160	—	6
B70	2	FLK-BLV cells, $2.4 \times 10^8$	Not tested	—	5

a)The antigen titer (p24) of the inoculum was determined by the ID test after the concentration 100 times. b)Bovine embryonic thymus cell cultures persistently infected with BLV. c)Cell free material. d)Short-term culture of  $1.5 \times 10^9$  tumor cells obtained from a cow with EBL. e)Short-term culture of  $1.5 \times 10^9$  PLL obtained from animal B9. f)Fetal lamb kidney cells persistently infected with BLV.

## BLV.

*Inoculation of BLV-infected non-PL cattle with PLL:* Two (B8 and B32), 1 (B2) and 2 (B3 and B4) cattle which had been inoculated with cell-free virus 4, 16 and 22 months earlier, respectively, and had not shown PL were inoculated with  $7 \times 10^9$ ,  $7 \times 10^9$  and  $4 \times 10^9$  PLL from B9 (Fig. 3). Two of them (B2 and B8) showed a sharp increase in the number of PBL 21 dpi. B8 maintained elevated PBL counts ranging from 17,000 to 26,000/ $\mu$ l until 16 mpi when the animal was sacrificed. B2 was killed 28 dpi when the number of PBL had increased to 14,500/ $\mu$ l. The remaining 3 animals (B3, B4 and B23) showed a gradual progressive increase in the number which reached the maximum 2 to 6 months after the inoculation. In contrast, 3 calves (B68, B70, and B71) which had been inoculated with cell free virus or FLK-BLV cells 5 to 6 months earlier, and had not shown PL were inoculated with  $5 \times 10^9$  PBL obtained from a healthy cow. These animals showed no increase in PBL counts for 5 months.

*Hematological examinations:* An increase or decrease in the percentage of lymphocyte was always accompanied by an increase or

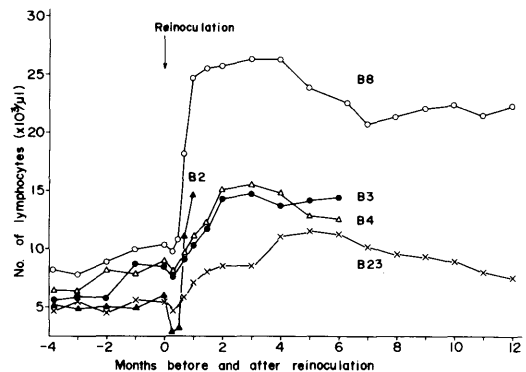


Fig. 2. Changes in number of PBL in cattle inoculated with PBL collected from donor B9. B5 and B21 were inoculated with  $2.4 \times 10^9$  PBL and B6 and B22 with  $2.4 \times 10^7$  PBL. The number of PBL of each test was plotted until 2 mpi and thereafter the average number of 2 to 4 tests per month was shown.

decrease in the number of leukocytes in all cases. The ratio of lymphocytes which was 36 to 70% before inoculation increased to 85 to 97% at the highest with an average of 88% in cattle which developed PL, while it remained in the normal range in cattle that failed to develop PL. The percentage of lymphocytes in one calf, B22, returned to the preinoculation level after the animal recovered from PL.

*Antibody responses in cattle inoculated with PLL or cell-free virus:* The rise in antibody to BLV was significantly earlier in cattle inoculated with PLL than in cattle inoculated with cell-free BLV (Table 3). Similarly, the highest antibody titers of individual animals showed a tendency to be higher in cattle inoculated with PLL.

*Histopathological changes in cattle with PL:* One animal (B9) died of emaciation with posterior paresis at 44 mpi. Histopathologically lymphosarcomatous lesions were present in the abomasum, heart and epidural fatty tissue of the spinal cord. Details were described previously [21]. In the other animals, slight swelling of the lymph nodes and hyperplasia of lymphocytes in the lymph nodes were consistently seen, but no tumorous changes were found. Details of the histological changes will be described elsewhere.

#### DISCUSSION

It has been considered that induction of PL by infection with BLV is influenced by the host's genetic factors [1, 2, 32]. In this study, we found that experimental PL could be induced rapidly by inoculation of a large number of PLL in every animal even if it had a different genetic make-up. Also, PL could be induced by inoculation with PLL that were collected from different donors. These data suggest that PL can be induced across the genetic barrier if a large number of PLL is inoculated and that different strains of BLV may have a similar ability to induce PL.

The degree of PL was dependent on the number of PLL inoculated. Also, it was hard to induce PL by inoculation of PBL collected from donors with inapparent PL or without PL. It is known that PLL produce a larger number of infective BLV than PBL of non-PL cattle [22, 26, 33, 36]. Therefore, the number of PBL containing BLV pro-

Table 3. Antibody responses of cattle inoculated with PLL or cell-free virus

Inoculum	No. of cattle tested	Time of rise in antibody (dpi) <sup>a)</sup>	Highest antibody titer
PLL	13	11–28 (17.46±2.71) <sup>b)</sup>	8–64 (21.54± 9.33)
Cell-free virus	8	18–42 (31.38±7.33)	1–32 (12.13±10.43)

a) Days post inoculation

b) Average and 95% confidence limit

virus might have an important role in induction of PL. In other words, the number of infective viruses in the inoculum may have a definite effect on the induction. However, calves failed to develop PL after inoculation with short-term culture fluids obtained from such a large number of PLL as 50 times that which could induce PL or 200 ml of culture fluid of FLK-BLV cells containing about 10<sup>5</sup> syncytia-forming units of BLV/ml. The short-term culture fluid, as well as other cell-free viral suspensions, contained a detectable amount of viral core protein (p24) antigen. Also, numerous lymphocytes producing many C-type virus particles were observed electron microscopically in short-term cultures (data are not shown) as reported previously [31, 43]. Therefore, failure to develop PL after inoculation with cell-free virus may not be due to a shortage in the amount of virus inoculated.

On the other hand, the finding that antibody response to BLV was earlier and higher in cattle inoculated with PLL than in cattle inoculated with cell-free virus indicates that propagation of a larger number of viruses occurred at an earlier time in cattle inoculated with PLL. Simultaneous inoculation of a large number of normal PBL and the virus failed to show enhancement of infection and development of PL (data are not shown). Therefore it is suggested that the lymphocyte-associated virus could initi-

ate infection *in vivo* more easily and more effectively than cell-free virus.

Development of PL was also observed in BLV-infected non-PL cattle after inoculation of PLL but not after inoculation of normal PBL. It has been shown that cattle infected chronically with BLV produce neutralizing antibody which suppresses maturation of BLV [3,10, 37], and nonimmunoglobulin plasma protein which inhibits expression of the BLV genome [19, 20]. Therefore, induction of PL in the non-PL cattle after inoculation of PLL may not be due to released cell-free virus. These findings suggest the possibility that infection with BLV is effectively established by interaction between donor lymphocytes and recipient lymphocytes. Such interaction might not occur between lymphocytes and fibroid cells such as BLV-FLK cells. It has been shown that lymphatic cells transfer to and settle effectively in the lymphatic tissues with the aid of a homing receptor [15]. Furthermore, HTLVs which have great similarity to BLV [7, 46] infect and transform human lymphocytes more easily by cocultivation with infected lymphocytes than cell-free virus, *in vitro* [16]. An early and rapid decrease in lymphocytes from the peripheral blood of recipient cattle after inoculation of PLL as shown in this study may be one of the phenomena of the interaction. An immunological booster effect that was observed in sheep immunized with BLV after challenge inoculation with a small number of infected sheep lymphocytes [25] also suggests the interaction between donor and recipient lymphocytes. The interaction may occur only with freshly prepared lymphocytes not with cultivated lymphocytes. Rapid and effective development of PL has been observed in an experiment using freshly prepared PLL [17], but not in a test using cultivated PLL [34].

Although, the mechanisms of development of PL are not known, a hypothesis of

transformation by HTLVs [42], in which a protein encoded by the viral genome acts by *trans*-activation to promote transcription of both viral and host cellular genes, might help our understanding of the pathogenesis of PL. A factor that *trans*-activates long terminal repeat of BLV has been found in cells infected with BLV [8, 40]. The factor may activate a gene(s) controlling cellular growth. Therefore, expression of BLV genome and propagation of BLV may play an important role in the development of PL in an early stage of infection [26]. It is known that expression of viral genome is inhibited in a late stage of infection with BLV [23, 24]. In contrast, limited expression of BLV genome in the chronically infected animals is suggested by life-long persistence and occasional increase in antibody levels to BLV in the animals [28]. Therefore, further investigation on expression of BLV genome *in vivo*, development and persistence of nonimmunoglobulin plasma protein which inhibit expression of the BLV genome [19, 20] and role of *trans*-acting factors seems to be important for understanding the pathogenesis of PL.

It is thought that tumorous transformation of cells by BLV occurs at only a very low rate. Therefore PL, which represents an increase in infected lymphocytes [26], may offer a high chance for transformation of the cells. In this study, one animal that had very marked PL died showing tumorous changes 3 years and 7 months after inoculation with PLL. Thus, it may be necessary to reconsider the role of PL in development of the tumor.

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## 要 約

牛白血病ウイルス接種により起こる牛の持続性リンパ球増多症とその継代：甲野雄次・泉対 博・新井啓五・松田 泉<sup>1)</sup>・石野清之（家畜衛生試験場，<sup>1)</sup>農業環境技術研究所）——牛白血病ウイルス（BLV）抗体を持ち、持続性リンパ球症（PL）を示した2頭の牛の $1.5 \times 10^8$ 又は、 $5 \times 10^9$ 個のリンパ球をそれぞれ1頭の子牛に接種した。接種後14及び23日目にリンパ球の急激な増加が起こり、その数は72及び57日後に最高値（29,000及び52,000個/ $\mu$ l）に達した。リンパ球数はその後減少したが、PLは観察期間中持続した。PLはPL時のリンパ球（PLL）の接種により常に起こり、連続継代が可能であった。PLLの接種量と発生したPLの程度は平行関係にあり、PLを示さなくなった時期のPBLはPLを起こさなかった。PLLをBLV抗体陽性・PL陰性牛に接種した時もPLは発生した。一方、PLは細胞フリーウイルスの接種では発生せず、抗体の産生も遅かった。これらの事象はBLVの感染が感染リンパ球により効率的に起きること、この感染がリンパ球間の相互接触により起こる可能性、及びBLVの実際の感染量がPLの発現に重要な意味を持つことを示していると解釈される。