

## Prevalence of Lymphocyte Nuclear Pockets in Holstein-Friesian Dairy Cattle Infected with Bovine Leukemia Virus in Korea

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**ABSTRACT** : The integral relationship between the occurrence of lymphocyte nuclear pockets (LNPs) and BLV-infection was examined in Holstein-Friesian dairy cattle in Korea. Transmission electron microscope (TEM) was used to detect LNP in peripheral blood lymphocytes. Morphologically, the membranes of LNP were composed of two layers of double nuclear membrane. The full thickness of LNP membranes including inner and outer nuclear membrane was 60 to 70 nm. LNP prevalence was different according to the bovine leukemia virus (BLV) infection status; in BLV-seropositive cattle, LNP prevalence was 48.4% and in BLV-seronegative cattle prevalence was 5.9%. Moreover, even in seropositive animals, leukemic group was the highest at 70% positive among the groups, followed by suspect group (42.4%) and aleukemic group (23.1%). Consequently, the numbers of LNP were increased in proportion to increase of the numbers of leukocytes among BLV-seropositive cattle. The numbers of LNP per lymphocyte were increased in BLV-seropositive cattle compared with seronegative cattle. The mean numbers of LNP per 100-lymphocytes were 0.35, 0.77, 1.64 and 4.7 in BLV-seronegative, BLV-seropositive aleukemic, suspect and leukemic groups, respectively. Thus, it is reasonable that LNP test can be used as the one of the diagnostic criteria of BLV infection. (*Asian-Aust. J. Anim. Sci.* 2005, Vol 18, No. 6 : 879-883)

**Key Words** : BLV, EBL, Bovine, Enzootic, Pocket, Leukemia, Leukosis

### INTRODUCTION

Enzootic bovine leukosis (EBL) caused by BLV infection is also referred as bovine lymphosarcoma, or leukosis of adult cattle. The cytoplasmic area in lymphocytes which is enclosed by nuclear membrane was identified by Smith and O'Hara (1967). This nuclear membrane is continuous with the extranuclear cytoplasm in human lymphocytes. Ring-shaped projection, which appeared isolated from the nucleus, was continuous with the nucleus in a deeper plane of the section. These observations led to the conclusion that this structure should be termed "nuclear pockets" and that they were formed by finger-like invagination of cytoplasm into the nucleus. Lymphocyte nuclear pocket (LNP) structures have been observed in bovine leukosis cases (Miller et al., 1969). Several ultrastructural studies of buffy coat lymphocytes from cattle with EBL indicated that LNP was a morphologic marker specifically related to this disease (Weber et al., 1969 and 1980).

Following the first serological detection of EBL in 1982 in Korea (Jun et al., 1982), it has now become clear that BLV infection occurs all over the country; with over 54.2% of Holstein-Friesian dairy cattle found to be BLV-seropositive (Suh, 2004). We have interested in electronmicroscopic study and have observed the LNPs in

peripheral blood lymphocytes of naturally BLV-infected cattle. It was of importance to compare LNP incidence not only between BLV-seropositive cattle and -seronegative cattle, but also between in aleukemic and leukemic cattle among BLV-seropositive cattle.

### MATERIALS AND METHODS

#### Animals

Ninety-eight Holstein-Friesian dairy cattle were used in this study: BLV-noninfected (34 heads) and naturally infected (64 heads). They reared in Chungnam-Do, Chungbuk-Do and Kyeonggi-Do of Korea. All examined cattle were female and over three years old.

Cows were classified in the base of the presence of BLV-antibody and the number of leukocytes: aleukemic, below 10,000 leukocytes with below 60% lymphocytes; suspect, 10,000-18,000 with 60-75% lymphocytes; and leukemic, over 18,000 with over 75% lymphocytes (Bendixen, 1965). Consequently, cattle were classified into four groups: group 1, BLV-seronegative cattle; group 2, BLV-seropositive and aleukemic status of leukocytes; group 3, BLV-seropositive and suspect status of leukocytes; group 4, BLV-seropositive and leukemic status of leukocytes.

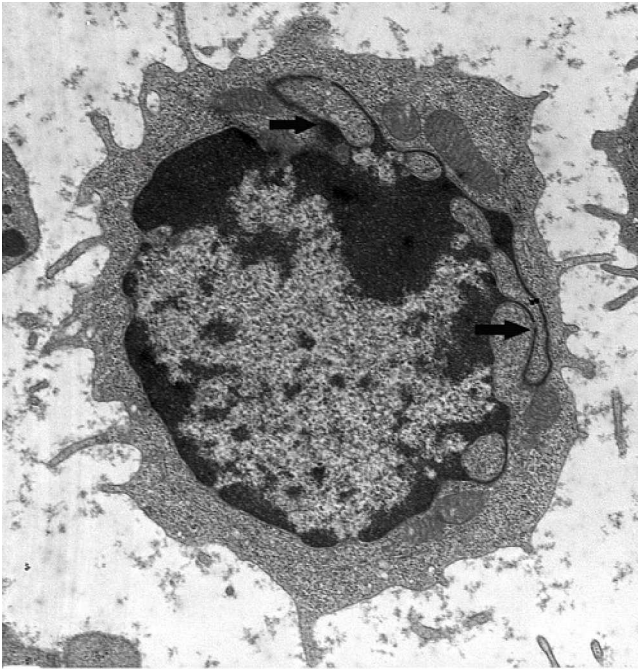
#### Leukocyte count and BLV antibody test

Blood was collected from tail vein into the vacutainer containing K3-EDTA. Leukocytes were enumerated using automatic blood cell counter (Vet ABC, Micros analyzer, France). For differential count of leukocytes, Giemsa stained-slides were observed under microscope (Straub,

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**Figure 1.** Lymphocyte. Note the loop or ring-shaped multiple lymphocyte nuclear pockets (LNPs) (arrows) containing cytoplasmic material.

1978). To determine BLV infection, presence of serum antibodies to BLV envelope glycoprotein 51 (GP51) and internal protein 24 (P24) were tested using commercially available agar gel immunodiffusion (AGID) kit (Synbiotics, Lyon, France) (Roberts et al., 1989).

#### Sample preparation for electron microscope

Peripheral blood lymphocytes were isolated by Histopaque 1077 (Sigma, USA) density gradient centrifuge. Five milliliters of blood were centrifuged at  $500\times g$  for 5 minutes and the buffy coat was harvested. The buffy coat was mixed with same volume of Histopaque 1077 and centrifuged at  $800\times g$  for 30 minutes. After centrifugation, mononuclear cell layers were collected in new tubes (Stock et al., 1972; Baliga et al., 1977; Joo et al., 2003). Thin sections were made with collected cells according to the previous reports (Weber et al., 1969, 1973; Hwang et al., 2002; Sen et al., 2004). Isolated lymphocytes were fixed for 20 minutes as a pellet by adding one ml of 2.5% glutaraldehyde (GA). After GA fixation, the cells were pelleted, washed, and fixed in 1% osmic acid. These cells were embedded in Eponate 12 kit (Pelco 18012) using propylene oxide and sectioned with an ultramicrotome (Leica LG Germany), and double-stained with uranyl acetate and lead citrate. Finally, stained sections were examined under a transmission electron microscope (TEM) (Hitachi 5700, Japan).

#### LNP examination

For LNP examination, 100 lymphocytes per cattle were



**Figure 2.** Membrane of lymphocyte nuclear pocket (LNP). Note the four layers; two inner layers (arrowheads) and two outer layers (arrows).

inspected. Criteria for LNP judgment were designated as follows according to the previous report: negative, no LNP-possessing lymphocyte; suspect, 1 LNP-possessing lymphocyte; positive, more than 2 LNP-possessing lymphocytes per 100 lymphocytes (Weber et al., 1974).

## RESULTS

Combined results of serological test and leukocyte count were as follows: the number of cows in group 1 was 34 heads (35%); group 2 was 13 heads (13%); group 3 was 28 heads (29%); group 4 was 23 heads (24%).

In TEM test, the profiles of LNPs were predominantly of the ring or loop types that extended from the usual contour of the nuclei and enclosed cytoplasmic substance (Figure 1). LNPs were made by evagination of the nuclear membrane that partially surrounded bits of cytoplasm. The structure of LNPs was formed with nonfold, cup-shaped evagination of the nuclear membrane into the cytoplasm. Thus the LNPs usually have similar components with the surrounding cytoplasm. Infrequently, the center of LNP was filled with clusters of electron opaque particle and with variable sized loops. However, some projections enclosed one or more vesicular structures occasionally containing amorphous granular material but no cytoplasmic elements. Sometimes ring shaped pockets with no visible nuclear attachment were seen in the cytoplasm.

The membrane of LNPs was relatively uniform in structure and consisted of two sheets of typical double-

**Table 1.** The number of lymphocytes with lymphocyte nuclear pockets (LNPs) per 100 lymphocyte in the bovine leukemia virus (BLV) antibody-positive and -negative cattle

Serology	Groups	Results of LNP test							Total
		Negative	Suspect	Positive				Subtotal	
		0 <sup>1</sup>	1	2 <sup>2</sup>	3	4 to 8	>9		
BLV- negative	Group 1	22	10	1	1	0	0	2	34
		(64.7) <sup>3</sup>	(29.4)					(5.9)	(100)
BLV- positive	Group 2	10	0	2	0	1	0	3	13
		(76.9)	(0)					(23.1)	(100)
	Group 3	10	6	6	4	2	0	12	28
		(35.7)	(21.4)					(42.9)	(100)
	Group 4	2	5	1	3	7	5	16	23
	(8.7)	(21.7)					(69.6)	(100)	
	Subtotal	22	11	9	7	10	5	31	64
		(34.4)	(17.2)					(48.4)	(100)
Total		44	21	10	8	10	5	33	98
		(44.9)	(21.4)	(10.2)	(8.2)	(10.2)	(5.1)	(33.7)	(100)

<sup>1</sup> No. of lymphocytes with LNPs in 100 lymphocytes.<sup>2</sup> Cows which had more than two LNP-containing-lymphocytes were classified as LNP positive.<sup>3</sup> No. of heads (percent).**Table 2.** The number of lymphocyte nuclear pockets (LNPs) per lymphocyte in the bovine leukemia virus (BLV) antibody-positive and -negative cattle

Serology	Groups	Results of LNP test				Total
		1 <sup>1</sup>	2	3	>4	
BLV-negative	Group 1	12	0	0	0	12
		(100) <sup>2</sup>	(0.0)	(0.0)	(0.0)	(100)
BLV-positive	Group 2	10	0	0	0	10
		(100)	(0.0)	(0.0)	(0.0)	(100)
	Group 3	41	3	1		46
		(89.1)	(6.5)	(2.2)	(2.2)	(100)
	Group 4	90	14	3	1	108
	(83.3)	(13.0)	(2.8)	(1.0)	(100)	
	Subtotal	141	17	4	2	164
		(86.0)	(10.4)	(2.4)	(1.2)	(100)
Total		153	17	4	2	176
		(87.0)	(9.7)	(2.3)	(1.1)	(100)

<sup>1</sup> No. of LNPs in one lymphocyte. <sup>2</sup> No. of lymphocytes (percent).**Table 3.** Mean number of lymphocyte nuclear pockets (LNPs) per 100 lymphocytes in the bovine leukemia virus (BLV) antibody-positive and -negative cattle

Serology	Groups	Results of LNP test		
		No. of animal tested	Total No. of LNP detected	Mean No. of LNP/100 lymphocytes
BLV- negative	Group 1	34	12	0.35
BLV- positive	Group 2	13	10	0.77
	Group 3	28	46	1.64
	Group 4	23	108	4.70
	Subtotal	64	164	2.56
Total		98	176	1.80

layered nuclear membrane. Two inner nuclear membranes were located between two outer nuclear membranes. Two inner membranes were 38 to 44 nm in thickness and separated by a band of osmiophilic material and each outer nuclear membrane was 11 to 18 nm in thickness. The total

thickness of LNP membranes was 60 to 70 nm (Figure 2).

The prevalence of LNP was different according to the experimental groups. Group 4 was the highest in prevalence among four groups as 69.6%, while that of group 1 was the lowest as 5.9%. In BLV-seropositive cattle, prevalence of LNP was increased in proportion with the increase of the number of leukocytes in the peripheral blood (Table 1). Not only positive percentage of LNPs but also the number of lymphocyte having LNPs in group 4 was higher than other groups (Table 1). In five cattle among group 4, LNPs were detected in more than nine lymphocytes.

Table 2 shows pocket numbers in pocket positive lymphocyte. All cattle in the group 1 and 2 had only one LNP in one lymphocyte, but 10.9% and 16.7% of cattle in the group 3 and 4 had over two LNPs in one lymphocyte, respectively. The mean pocket numbers per 100 lymphocytes were 0.35, 0.77, 1.64 and 4.7 in group 1, 2, 3 and 4, respectively (Table 3).

## DISCUSSION

Bovine leukosis is a lymphoproliferative disorder that has been divided into enzootic bovine leukosis (EBL) and sporadic bovine leukosis (SBL) (Bendixen, 1965). BLV, which is the causative agent of EBL, was discovered in 1969 by Miller et al. BLV is in the subfamily Oncovirinae, family Retroviridae and further classified into HTLV-BLV group (Chevallier et al., 1998). In most cases, BLV infection remains clinically silent (Burney et al., 1988) and only one-third of infected cattle develop persistent lymphocytosis (PL), a polyclonal expansion of B lymphocytes. After a latency period of 3 to 10 years, lymphoma, a monoclonal neoplastic transformation of infected B lymphocytes occurs in fewer than 5% of the infected cows (Matheise et al., 1992; Depelchin et al., 1989; Dequiedt et al., 1999).

In the past, measuring the number of lymphocyte in peripheral blood was used as a tool to diagnose EBL. However, the reason why the numbers of lymphocytes increase, was not clearly defined without assistance of virologic methods (Miller et al., 1972). In order to complement this problem, new method was required to identify whether the increase of the number of lymphocytes was caused by BLV infection or not; and as the result, a method that inspects LNP, which is a constituent of cell to be generated during abnormal splits and proliferation of lymphocytes, had been proposed (Smith and O'Hara, 1968; Miller et al., 1972; Weber et al., 1974). Results of several ultrastructural studies of buffy coat lymphocytes from cattle with EBL indicated that LNP is a morphologic marker specifically related to this disease (Weber et al., 1969; Pomeroy et al., 1977).

In this study, LNP denotes the nuclear membrane that protruded and expanded toward cytoplasm shaping a sac and also the nuclear membrane that constitutes LNP has been always overlapped in a duplicated manner (Bloch et al., 1975). LNP was easily identified due to its peculiar shape when examined in electronmicroscopic level. LNP, which was observed in this research, was identical with the one that already reported (Weber et al., 1969). The membrane of LNP was 60 to 70 nm in thickness, and it was similar to previous reports (Smith and O'Hara, 1968; Miller et al., 1969; Bloch et al., 1975). Most projections were single loop of nuclear material that enclosed cytoplasm. Multiple pockets were noted in few cells; they were either distributed around the periphery of a nucleus or adjacent to one another in highly complex formation (Miller et al., 1969). These large, single or multiple vesicles within the nuclear loop suggested that the portions of cytoplasm had been pinched off by the nuclear sheet with secondary loss of cytoplasmic ribosomes and hyaloplasm. This is similar finding to the previous report (Smith and O'Hara, 1967).

There was a prominent difference in LNP expression between BLV-seropositive and -seronegative group (Table 1), and it also showed that the number of lymphocytes with LNP increased particularly in proportion to the increase of the number of leukocyte in peripheral blood (Table 2). In the group 1, the rate of LNP suspect cattle was high, while the rate of LNP positive cattle was low (Table 1). Although LNP exists in the normal cattle that have not been infected with BLV, the number of lymphocytes having LNP was less than 2%. In consequence, it was judged that BLV infection be suspected only when LNP existed more than 2% of lymphocytes, then it was thought that the LNP test can be used as a diagnostic index for EBL.

The number of LNP was matched with the report of Weber (1969, 1974 and 1980). That is, the total number of LNP per 100 lymphocytes in the group 2 was 0.77 and it was almost same expression of LNP (0.75) as reported by Weber et al. (1973). Meanwhile, it was confirmed that multiple pockets exist in a lymphocyte of the BLV-infected cattle, and this finding was congruous with the existing reports (Smith and O'Hara, 1968; Miller et al., 1969). Particularly, in case of group 4, the prevalence of lymphocytes which had more than four LNP was 57% (Table 1), therefore it was thought to be an significant marker.

As the result of LNP test, there was a difference on LNP expression between BLV-seropositive and -seronegative Holstein-Friesian dairy cattle, and also among the seropositive groups, the proportion of LNP-positive cattle was increased in proportion with the increase of the leukocyte number in peripheral blood. Therefore, method of LNP detection may be used as a compensatory test to PCR for the early diagnosis of the BLV infection. It would be important to identify the LNP-positive lymphocytes by flow cytometry using monoclonal antibodies specifically reactive with bovine leukocyte differentiation molecules to clarify the pathogenesis of EBL and formation of LNP.

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