Bovine Leukemia virus titer and leukocyte population associated with mastitis in peri-parturient dairy cows

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ABSTRACT

We examined the association of postpartum mastitis with virus titer and leukocyte population of Bovine leukemia virus (BLV) infected cows monitoring sixteen BLV infected cows for 3 months after calving. Five cows developed mastitis (Mastitis Group) and 11 cows without mastitis served as a control group (Control Group). Flow cytometric analysis of peripheral blood leukocyte population exhibited higher number of MHC class-II+CD14- cells in both groups without significant difference between groups. $CD3^+$, $CD4^+$, $CD8^+$, and $CD335^+$ cell numbers in Mastitis Group were lower than the Control Group around the time of calving, but significant difference was observed only for CD335+ cell number. Virus titer in blood significantly increased in Mastitis Group after calving compared to pre-calving titer. These results suggested that lower number of peripheral blood T cells in Mastitis Group cows might have heightened susceptibility to mastitis. In addition, Mastitis Group cows had higher peripheral blood BLV, which might have spread to surroundings.

INTRODUCTION

Bovine leukemia virus (BLV) belongs to retrovirus family similar to human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV). Although BLV infection remains subclinical in the majority of cattle, some cows progress to persistent lymphocytosis (PL) with an expansion of B lymphocytes. Some cows with PL further develop B cell tumor and then enzootic bovine leukosis (EBL).[15,20] Cows with advanced BLV infection showed decreased

Table 1. *Clinical course and pathogenic bacteria in Mastitis group.*

Case No.	Age	Clinical sign	Temperature on first examination (℃)	Treatment period (days)	Pathogenic bacteria
Mastitis-1	3.53	Severe	39.8	3	ND
Mastitis-2	3.19	Mild	39.0	2	*Klebsiella*
Mastitis-3	6.90	Severe	39.0	3	*Streptococcus*
Mastitis-4	6.80	Mild	39.0	3	*Staphylococcus aureus*
Mastitis-5	5.98	Mild	38.7	9	Yeast

Severe : Udder swelling was indurate and unelastic with a little lactation and aorexia.
Mild : Udder swelling was elastic and unindurate.
ND: Not determined.

immune function, thus reduced ability of natural recovery from *Trichophyton verrucosum*.[4] Dairy cows become susceptible to infectious diseases during the periparturient period due to decreased immune function.[11,19] This periparturient immunosuppression makes BLV infected cows vulnerable to develop EBL and heightens the possibility of virus spreading to other cows by increased peripheral blood virus.[5,16] In addition BLV infected cows in the periparturient period have immunological disturbances with increased peripheral B-cell number. It may further heighten the susceptibility of cows to other infectious diseases.[9,21] BLV infection is speculated to affect mastitis. In fact, BLV was detected in the mammary glands of mastitic cows[23] and BLV infected cows exhibited higher somatic cell counts in milk.[22] Economic losses due to the BLV infection can come from reduced milk production, because the milk yield was higher in healthy cows compared to BLV seropositive cows.[14]

There has been no information about the virus titer in the peripheral blood of BLV infected cows during the periparturient period and its association with mastitis development. In this study we examined the virus titer and leukocyte populations in the peripheral blood of BLV infected cows around the time of calving, and their association with clinical mastitis.

MATERIALS and METHODS

Animals

Sixteen healthy Holstein cows positive for BLV by nested-PCR 12 kept at the same farm were used. Among them five cows developed mastitis within 3 months after calving, and they were grouped as Mastitis Group. They were diagnosed by California Mastitis Test (CMT) score and udder swelling. Clinical signs were severe in three cows, and mild in two cows.

One cow had high fever on the first medical examination. Milk samples from five cows were kept on sheep blood agar 24 to 48 hours at 37. After incubation, pathogenic bacteria of causing mastitis were identified in four cows (Table1). The remaining 11 were clinically healthy and they were grouped as Control Group. Peripheral blood samples were obtained twice; once within 4 weeks before calving (17.4 +/- 1.6 days before calving, mean +/- sem) and once at 1 to 6 weeks after calving (20.6 +/- 2.0 days after calving). The age of cows at the time of enrollment was 5.3 +/- 0.8 and 4.5 +/- 0.4 years for Mastitis Group and Control Group, respectively. There was no difference in their age. The pre-calving sampling date was 12.0 +/- 3.7 and 19.8 +/- 1.1 days before calving for Mastitis Group and Control Group, respectively. The post-calving sampling was conducted at 23.0 +/- 6.1 and 19.6 +/- 1.2

Table 2. *Antibodies used for immunostaining of peripheral blood mononuclear cells.*

Antigen	MAb clone	Isotype	Specificity	Source
CD3	MM1A	IgG1	Pan T cell	VMRD
CD4	CACT138A	IgG1	Helper/inducer	VMRD
CD8	BAQ111A	IgM	Cytotoxic T cell	VMRD
CD335	MCA2365	IgG1	NK cell	AbD
WC1-N1	B7A1	IgM	γδT-cell	VMRD
MHC Class II	CAT82A	IgG1	Class II major histocompatibility complex	VMRD
CD14	MY4	IgG2b	Monocyte	Coulter

VMRD=VMRD, Inc. (Pullman, WA, U.S.A).
Coulter=Becman Coulter (Tokyo, Japan).
AbD=AbD Serotec (Oxford, U.K.)
The original concentration of the MAb solution was 1 μg/ml.

days after calving for Mastitis Group and Control Group, respectively. The history of mastitis treatment in the previous lactation was found in two and one cow, for Mastitis Group and Control Group, respectively. Blood samples were obtained either from the caudal or jugular vein into EDTA-2K and heparin Na containing tubes.

BLV Titer

BLV titration was conducted by syncytium assay. Peripheral blood mononuclear cells (PBMC) were isolated using Percoll (Amersham Bioscience, U.S.) separation. PBMC (5 x 105) and CC81 cell (1 x 105) were placed into a well in 24-well plate and incubated at 37 °C with 5 % CO2 for 72 hours. Cells were stained with Giemsa and the number of syncytium, which contained more than 5 nuclei, was counted for each well. Three wells were used for each PBMC sample and the data were shown as an average of three wells.

Flow Cytometry

Total white blood cell (WBC) number was determined by automated cell counter (CelltacαMEK-6358, Nihon Kohden, Tokyo, Japan). The expression of cell surface antigen on white blood cells was determined by flow cytometry as previously reported.18 Monoclonal antibodies were used to determine the following cell surface antigens; CD3, CD4, CD8, CD335, WC1-N1, MHC class-II, and CD14. The specificity and source of these antibodies are shown in Table 2. The surface antigen positive cell number was calculated based on the percentage of each antigen positive cell determined by flow cytometry (FACScan, Becton Dickinson, USA) and the total number of lymphocytes obtained from cell counter.

Statistical Analysis

Difference between two groups in syncytium number and leukocyte population for pre- and post-calving was determined using Mann-Whitney's U test. Difference in syncytium number and leukocyte population between pre- and post-calving for each group was analyzed using Wilcoxon rank sum test. $P < 0.05$ was considered significantly different.

RESULTS

There was no significant difference between two groups in pre-calving samples. Although the titer in Control Group did not differ between pre- and post-calving, Mastitis Group showed significant increase in virus titer after calving (Table 3).

The numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in Mastitis Group were significantly lower in pre-calving sampling compared to Control Group. After calving the number of $CD335^+$ cells in Mastitis Group was signifi-

Table 3. *Peripheral blood leukocyte profile and BLV syncytium.*

		Mastitis Group (n=5)		Control Group (n=11)	
		Pre	Post	Pre	Post
WBC	(×100/μl)	147.00±24.49	126.60±27.33	151.36±18.56	142.27±22.22
PBMC	(×100/μl)	91.91±19.49	76.90±21.29	103.19±18.88[b]	84.98±24.89[b]
Granulocytes	(×100/μl)	55.09±9.93	49.70±7.28	48.17±4.59	57.30±7.91
$CD3^+$	(×100/μl)	9.68±1.73*	9.11±1.84	16.24±1.62[b]*	11.45±1.35[b]
$CD4^+$	(×100/μl)	2.98±0.53*	3.22±0.93	7.07±1.09[b]*	4.65±0.41[b]
$CD8^+$	(×100/μl)	2.23±0.27*	2.50±0.62	4.39±0.62*	3.46±0.88
$CD14^+$	(×100/μl)	10.32±2.81	8.93±2.51	9.81±1.06	7.37±1.39
$CD335^+$	(×100/μl)	1.26±0.41[a]	0.75±0.33a*	2.09±0.42	1.37±0.19*
WC1-$N1^+$	(×100/μl)	2.81±0.80	2.04±0.72	3.84±0.51[b]	2.72±0.36[b]
MHC class-II+CD14-	(×100/μl)	70.80±21.14[a]	52.58±19.92[a]	75.76±18.49	62.77±24.08
Syncytium	(/well)	508.80±149.76[a]	1478.20±426.62[a]	550.64±156.55	525.73±233.49

cantly lower than that in Control Group. The numbers of PBMC, $CD3^+$, $CD4^+$ T cells and WC1-$N1^+$ cells in Control Group decreased significantly after calving compared to pre-calving samples. The number of $CD335^+$ cells and MHC class-II+CD14- cells in Mastitis Group decreased significantly after calving compared to that in pre-calving sample (Table 3).

The percentage of $CD4^+$ T cells in Mastitis Group were significantly lower in pre-calving sampling compared to Control Group. After calving the percentage of $CD335^+$ cells in Mastitis Group was significantly lower than that in Control Group. The percentage of $CD335^+$ cells in Mastitis Group decreased significantly after calving compared to that in pre-calving sample (Table 4).

Although causes of five cows' mastitis were variable, using Smirnov-Grubbs test in both pre-calving and post-calving Mastitis group, there was no outlier in the number of syncytium and population.

DISCUSSION

The objective of this study was to clarify the difference in BLV cows between with (Mastitis Group) and without (Control Group) mastitis during the periparturient period when immune function is suppressed. We focused on the difference in virus titer and leukocyte population in Mastitis Group and Control Group.

Periparturient dairy cows have decreased number of T cells and their cytokine production skews toward Th2 type by expressing more mRNA of IL-4 and IL-10. This may contribute to immunosuppression and makes cows more vulnerable to mastitis.[11,19] In the present

Table 4. *Percentage of peripheral blood mononuclear cell profile*

		Mastitis Group (n=5)		Control Group (n=11)	
		Pre	Post	Pre	Post
$CD3^+$	(%)	12.28±2.54	15.67±3.65	20.47±3.44	19.95±3.26
$CD4^+$	(%)	3.37±0.43*	5.06±1.49	9.16±1.62*	8.76±1.61
$CD8^+$	(%)	3.04±0.86	4.93±1.78	5.26±0.92	5.41±1.05
$CD14^+$	(%)	16.48±7.76	14.44±3.59	11.89±1.94	13.92±2.95
$CD335^+$	(%)	1.68±0.54[a]	1.00±0.28[a]*	2.24±0.33	2.32±0.34*
$WC1\text{-}N1^+$	(%)	3.61±1.03	3.06±0.84	4.39±0.66	4.75±0.78
MHC class-II+CD14-	(%)	69.28±10.46	59.81±9.08	65.09±5.37	60.65±6.15

Data are expressed as Mean±S.E.
Pre: Precalving. Post: Postcalving.
a,b: Significant difference between the same letters (P <0.05).
**: Significant difference between groups (P <0.05).*
Mann-Whitney's U test and Wilcoxon rank sum test were used.

study, Mastitis Group cows exhibited lower number of CD3+, CD4+, and CD8+ T cells compared to Control Group in the pre-calving samples. Decreased T cell number prior to calving might have increased susceptibility of cows to mastitis by reducing responsiveness of T cells to infection.

We previously reported that BLV infected cows had higher number of B cells compared to non-BLV at the time of parturition. Peripheral and mammary gland mononuclear cells of BLV infected cows expressed more IL-4 and IL-10 mRNA after parturition compared to non-BLV infected cows [9]. IL-4 and IL-10 are known to mediate humoral immunity and suppress production of cytokines associated with cell-mediated immunity such as IL-12 and IFN-γ.[17] Domination of humoral immunity makes BLV cows more prone to progressing PL or EBL.[8] Cows with PL is reported to have reduced immune responsiveness and T cell function.[7] Thus periparturient BLV infected cows are more prone to develop mastitis as well as EBL.

The number of B cells in the peripheral blood increases in BLV infected cows as the disease progresses.[15,20] Since B cells and monocytes express MHC class-II in the peripheral blood, CD14-negative MHC class-II positive cells are recognized as B cells.[1] The percentage of B cells among peripheral MHC class-II positive cells from BLV infected cows has been shown to increase considerably more than uninfected cows.[3] In our previous study, clinically healthy BLV infected dairy cows showed high number of peripheral blood MHC class-II+CD14- B cells,10 which was similar to the number of MHC class-II+CD14- B cell in Control Group of the present study. There was no significant difference in MHC class-II+CD14- B cell number between Control Group and Mastitis Group. There was a possiblity that development of mastitis was not associated with the peripheral blood B cell number.

Increase in BLV titer is associated with higher risk of BLV spreading. Especially periparturient dairy cows with BLV have lower antibody titer against BLV, thus increased shedding of virus into blood, milk, and other body fluid.[5] The syncytium number in the present study did not show significant difference between groups both at pre- and post-calving. However, Mastitis Group cows showed a significant increase in syncytium number after calving compared to pre-calving number. Mastitis Group cows had lower number of $CD335^+$ NK cells after calving compared to Control Group. NK cells play an important role in the early stage of

immune response by killing virus or tumor cells using their cytotoxic ability as well as cytokine production.[2] NK cells indirectly respond to bacterial infection via activating macrophages and dendritic cells.[6] Decreased NK cell function is reported in HIV infected patients.[13] Therefore, Mastitis Group cows might have increased BLV in the peripheral blood due to lower cell mediated immunity of NK cells.

In the present study, we found that BLV cows with lower number of T cells in the pre-calving period were prone to develop clinical mastitis after calving and increase in blood BLV titer thus risk of virus spreading.

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