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Effect of *Escherichia coli* and *Staphylococcus aureus* on the *In vitro* Response of Peripheral Blood Mononuclear Cells Isolated from Holstein Dairy Cows had Ketosis or Milk Fever of Field Cases

[1]H. Ohtsuka, [1]N. Kanikawa, [2]K. Kudo, [2]F. Kyutoku, [3]S. Kakinuma, [4]M. Kohno,
[1]Y. Maeda and [1]T. Tanabe
[1]School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan
[2]Ishikari Agricultural Mutual Relief Association, Ebetsu, Hokkaido 067-0055, Japan
[3]Kakinuma Veterinary Hospital, 200-1 Kodama, Kodama-Chou, Honjou, Saitama 367-0212, Japan
[4]Miyagi Prefectural Federated Agricultural Mutual Aid Association, Shiroishi, Miyagi, 989-0731, Japan

Abstract: The expression of cytokines mRNA, including Interleukin (IL)-2, IL-4 and Interferon (IFN)-γ and lymphocyte proliferation stimulated *in vitro* with either *Escherichia coli* (*E. coli*) or *Staphylococcus aureus* (*S.A.*) by Peripheral Blood Mononuclear Cells (PBMC) obtained from Holstein dairy cows were examined after the onset of ketosis or milk fever of field cases during the lactation period. The cows were used 50 to 150 days after calving in this study and divided into three groups; seventeen cows with milk fever (MF Group, 94.9±8.6 days after calving), ten cows with ketosis (Ketosis Group, 96.3±10.2 days after calving) and twenty-seven cows with no clinical diseases (Control Group, 89.4±5.4 days after calving). PBMC were stimulated *in vitro* by soluble antigen of *E. coli* or *S.A.* together with recombinant IL-2. The numbers of the peripheral blood T cells and monocyte were lower in the Ketosis Group in comparison with those in the Control Group and a significant difference was found in the number of $CD3^{+}TcR1\text{-}N12^{+}T$ cells. There was a significantly lower lymphocyte proliferation stimulated with *E. coli* and *S.A.* in the Ketosis Group compared to that in the Control Group. Expression of IL-2, IL-4 and IFN-γ mRNA in PBMC stimulated with *S.A.* in the Ketosis Group was significantly lower than that in the Control Group, but these cytokine expression showed no group differences when PBMC were stimulated with *E. coli*. The present study demonstrated the onset of ketosis during the lactation period decreases reactivity of PBMC at least when they were stimulated with *S.A in vitro*.

Keywords: Cytokine, dairy cows, immune function, ketosis, milk fever

INTRODUCTION

Metabolic diseases of dairy cows, such as milk fever and ketosis, are well known to decrease lactation performance in the dairy cows. A cow with these metabolic problems exhibit lower immune function and tend to suffer from retained placenta, metritis, or endometritis. Odds ratios for the multiplicative effects of parturient paresis with hypocalcemia on incidence of veterinary-assisted dystocia, retained placenta, complicated ketosis and clinical mastitis were 7.2, 4.0, 23.6 and 5.4, respectively (Curtis *et al*., 1985). Previous investigation reported that hypocalcemia seems to interfere with the activation of immune cells in cows around the time of calving (Kimura *et al*., 2002). Authors suggested that immune suppression in cows with hypocalcemia was caused by a blunt signal transduction in immune cells following the insufficient stores of intracellular calcium. On the other hand, elevated circulating ketone bodies in the cows with ketosis may affect the immune system directly (Suriyasathaporn *et al*., 1999). High levels of Beta-Hydroxy Butyrate (BHB) inhibited bovine lymphocyte proliferation *in vitro* (Franklin *et al*., 1991). Although both periparturient hypocalcemia and ketosis cause decreased immune function, we observed that once cows had hypocalcemia around the time of calving, the lower T-cells recovered within 1 month, but lower T-cells remains until 3 months in the cows with displaced abomasums following lower serum cholesterol level (Ohtsuka *et al*., 2013), therefore the mechanism of immune suppression between two diseases might be different.

Mastitis, inflammation of the mammary gland, is considered the most costly disease of dairy cows worldwide (De Oliveria *et al*., 2000). *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S.A.*) are the most prevalent pathogens of bovine mastitis. However the

Corresponding Author: H. Ohtsuka, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan, Tel.: (81)-176-23-4371

pathogenesis and clinical symptoms of mastitis caused by both bacteria are considerably different (Oltenacu and Ekesbo, 1994). Mastitis caused by *E. coli* is usually acute and often develops serious problems such as a shock. Incontrast, *S.A.* mastitis often becomes chronic. One of the reasons for these differences is due to cytokine production. The higher expression of IFN-γ and IL-8 were observed in milk somatic cells isolated from mammary glands challenged with *E. coli* compared with *S.A.* challenge (Lee *et al*., 2006). Cytokines production stimulated by infected bacteria in the cows is different between Gram-positive and negative bacteria. Since immunosuppressive effects are different between cows with hypocalemia and cows with ketosis, it is possible that the immune cells obtained from cows with these metabolic diseases might differently respond to two types of bacterial challenges. There has been no report whether immune response stimulated with Gram-positive or negative bacteria is different between the cows had ketosis and the cows had milk fever during the lactation period. In the present study, we analyzed the lymphocyte proliferation and cytokine mRNA expression stimulated with either *E. coli* or *S.A. in vitro* using PBMC isolated from lactating Holstein dairy cows.

MATERIALS AND METHODS

Animals: Fifty-six Holstein dairy cows, housed in 7 dairy farms in Japan, were examined in this study. All cows were 50 to 150 days in lactation when they were used for this study. These cows were divided into three groups depending on the disease incidence; milk fever (MF Group, n = 17, 6.17±0.48 years old, 94.9±8.6 days in lactation), ketosis (Ketosis Group, n = 10, 6.43±0.73 years old, 96.3±10.2 days in lactation) and no clinical disease (Control Group, n = 29, 5.49±0.41 years old, 89.4±5.4 days in lactation). Both metabolic diseases were diagnosed and treated by veterinary clinicians after the disease onset in field. All cows recovered after the treatment and collected blood at lactating periods after recover. Blood samples were obtained only once from all cows. The number of cows in each herd is listed in Table 1. All cows in MF or Ketosis group did not have a complication of other diseases.

Analysis of leukocyte population: Blood samples were collected into tubes, which contained either heparin and disodium-EDTA. The total number of the White Blood Cells (WBC) in a blood obtained into disodium-EDTA containing tube was determined using a blood cell counter (Celltac MEK-6358, NIHON KOHDEN and Tokyo, Japan).

For the flow cytometric analysis of leukocyte populations, blood obtained into dipotassium-EDTA was hemolyzed using 0.83% ammonium chloride. After the hemolysis and washing with PBS (pH 7.2), WBC were re-suspended in cold PBS. Two-color flow cytometry was performed using these isolated WBC.

Table 1: Numbers of cows in each herd

Herd	Ketosis group	MF group	Control group
A	4	0	4
B	0	6	6
C	2	4	5
D	2	0	4
E	0	3	2
G	2	1	2
H	0	3	4
Total	10	17	27

Table 2: Monoclonal antibodies used for the immune staining of peripheral blood mononuclear leukocytes

Antigen	MAb clone	Iso type	Specificity	Source[1,2,3]
CD3	MM1A	IgG1	Pan T cell	VMRD
CD4	CACT138A	IgG1	Helper/inducer	VMRD
CD8	CACT80C	IgG1	Cytotoxic	VMRD
CD14	MY4	IgG2b	Monocyte	BC
CD335	MCA2365	IgG1	NK cell	Cerotec
TcR1-N12	CACT61A	IgM	γδ T-cell receptor	VMRD
MHC class-II	CAT82A	IgG1	Class II major histocompatibility complex	VMRD

[1]VMRD: VMRD, Inc. (Pullman, WA, U.S.A.); [2]BC: Becman coulter (Miami, FL, U.S.A.); [3]Cerotec: AbDCerotec (Martinsried, Germany); The original concentration of the MAb solution was 1 μg/mL

WBC (1×10^6) was incubated with monoclonal antibodies at 4°C for 60 min. We used monoclonal antibodies in order to detect CD3 (total T cells), CD4 (T helper cells), CD8 (T cytotoxic cells), CD14 (monocytes), CD335 (NK cells), TcR1-N12 (γδ T cells) and Major Histocompatibility Complex (MHC) class II (monocytes/B cells). The list of primary antibodies and the description of the working solutions are presented in Table 2. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgM and phycoerythrin-conjugated goat anti-mouse IgG (ICN Biomedicals, Costa Mesa, California, USA) at 4°C for 30 min. After washing the WBC with PBS, the flow cytometric analysis was performed using a Flow cytometer (Beckman Coulter, Miami, Fla, USA). Data from 10000 events/sample were analyzed using Flowjo and each leukocyte numbers were calculated by WBC and analyzed each surface antigen percentages in PBMC.

Analysis of lymphocyte proliferation and cytokines mRNA expression: For the analysis of lymphocyte proliferation and cytokines mRNA expression, *E. coli* and *S.A.* originally isolated from clinical cases of bovine mastitis were used as stimulants in this study. First, the Peripheral Blood Mononuclear Cells (PBMC) were isolated from the heparinized blood and 1×10^6 PBMC in RPMI 1640 medium supplemented with 2-ME and 10% fetal calf serum were placed in a 96-well plate for analysis of lymphocyte proliferation or 24-well plate for cytokines mRNA and incubated with 1 μg/mL of soluble antigen, either *E. coli* or 10 μg/mL of *S.A.*, together with 500 IU/mL of human recombinant interleukin (IL)-2 (PrimmuneInc, Kobe, Japan). For isolation of soluble antigen, *E. coli* and *S.A.* were disrupted by vortexing with glass beads and then

supernatant was isolated after centrifugation. Samples with only IL-2 stimulation were served as a control. After 7 days incubation at 37°C, 5 mg/mL of 3-(4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) was added to each well of 96-well plate. After dissolving dark blue formazan crystals, the Optical Density (O.D.) was measured using a micro plate reader at a wave length of 540 nm. The stimulation index was calculated using the formula below:

Stimulation Index (SI) = (O.D. of stimulated sample) / (O.D. of control) ×100

After 7 days incubation at 37°C, PBMC were re-suspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to analyze the cytokines mRNA expression of PBMC in the presence of soluble antigen. Analysis of cytokine mRNA levels was performed by a real-time PCR as described previously (Livak and Schmittgen, 2001; Maeda *et al*., 2013). Two micrograms of total RNA from each sample were used for synthesis of the first-strand of cDNA using oligo-dT primers (Invitrogen, Carlsbad, CA, USA) and superscript II reverse transcripts (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems., CA, USA) on an ABI prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The target DNA sequences were specifically amplified using the primers as previously designed (Riollet *et al*., 2001). The Ct values defined the threshold cycle of PCR, at which amplified products were detected. Fold changes in expression for the two groups (ΔΔCt) were calculated using the following formula:

Cytokine/β-actin = 2^ (- (A - B))

A : Cytokine Ct value containing soluble antigen-β-actin Ct value containing soluble antigen
B : Cytokine Ct value-β-actin Ct value

Ct values define the threshold cycle of PCR, at which amplified products were detected. Results are expressed as Ct values, where Ct is the difference in threshold cycles for target and β-actin as an internal control.

Statistical analysis: Statistical analysis for each parameter was performed using Tukey-Kramer's test in order to determine the differences among the three groups. The differences between groups were considered significant at $p<0.05$.

RESULTS

The numbers of lymphocyte subsets and monocyte in the peripheral blood leucocytes were determined for each group of cows, in order to determine the effect of diseases. Ketosis Group and MF Group showed lower lymphocytes and monocytes, as well as significantly lower TcR1-N12$^+$ T cells compared to the Control Group. Number of CD14$^+$ cell in Ketosis was significantly lower than that of Control Group (Table 3).

Table 3: The number of leukocyte subpopulation

Category	Ketosis group	MF group	Control group
CD3$^+$	6.6±1.1	7.2±0.7	11.7±1.0
CD4$^+$	3.8±0.7	3.5±0.4	4.7±0.5
CD8$^+$	1.9±0.3	2.3±0.4	3.1±0.2
TcR1-N12$^+$	1.3±0.2^a	1.8±0.2^b	4.0±0.6^{ab}
CD335$^+$	0.6±0.1	0.7±0.1	1.3±0.2
MHC class-II$^+$CD14$^-$	9.2±1.1	9.2±0.8	13.1±1.1
CD14$^+$	4.2±0.7^a	6.6±0.5^b	7.4±0.4^a

Data are expressed as the mean±S.E. (×10^2 cells/μL); *: Same letters indicate significant difference between groups within the same period ($p<0.05$)

Table 4: Levels of lymphocyte proliferation

Category	Ketosis group	MF group	Control group
S.A. + IL-2	65.8±9.3^a	91.6±12.4^b	110.6±13.8^a
E. coli + IL-2	32.9±8.7^a	61.1±11.7^b	66.2±9.9^a
IL-2	53.6±10.1^a	93.4±15.3^b	91.1±13.1^a

Data are expressed as the mean±S.E. (stimulation index); *: Same letters indicate significant difference between groups within the same period ($p<0.05$)

Table 5: Reactivity of cytokines mRNA expression

Category	Ketosis group	MF group	Control group
S.A. + IL-2 stimulation			
IL-2	1.59±0.41^{ab}	4.28±0.97^a	4.35±0.89^b
IL-4	0.98±0.23^a	1.51±0.31^b	2.84±0.86^a
IFN-γ	1.09±0.32^a	2.04±0.50^b	2.28±0.40^a
E. coli + IL-2 stimulation			
IL-2	2.93±0.94	2.23±0.95	2.17±0.47
IL-4	1.31±0.18	1.57±0.21	1.95±0.39
IFN-γ	1.09±0.23	1.56±0.55	1.87±0.48
IL-2 stimulation			
IL-2	0.12±0.04	0.20±0.06	0.45±0.12
IL-4	3.18±0.85	5.34±1.73	7.06±1.36
IFN-γ	2.40±0.94	1.79±0.68	1.87±0.30

Data are expressed as the mean±S.E. (cytokines/β-actin); *: Same letters indicate significant difference between groups within the same period ($p<0.05$)

Lymphocyte proliferation was significantly lower in the Ketosis Group, not only by the IL-2 stimulation but also by *E. coli* or *S.A.* in the presence of IL-2 compared to Control Group. The proliferation index in Ketosis Group was also lower compared to the MF Group. There were no differences in lymphocyte proliferation between MF Group and Control Group in the all culture conditions (Table 4).

After 7 days in the presence of *S.A.* with IL-2 stimulation, mRNA of IL-2 in Ketosis group was significantly lower compared to either MF Group or Control Group. In addition mRNA of IL-4 and IFN-γ were significantly lower than those in the Control Group. However, there were no significant differences in mRNA of IL-4 or IFN-γ in PBMC stimulated with *S.A.* between Ketosis and MF Group. In the presence of

E. coli, significant differences of the expression of IL-2, IL-4 and IFN-γ mRNA were not detected among three groups. No significant differences between MF Group and Control Group were observed in the all three cytokine mRNA expressions examined (Table 5).

DISCUSSION

Ketosis is known to increase the risk of clinical mastitis in the dairy cows (Oltenacu and Ekesbo, 1994). Hypocalcemia also greatly increases the risk of mastitis (Curtis *et al*., 1985) as well as the incidence of endometritis (Whiteford and Sheldon, 2005). The current study showed the lower peripheral γδ T cell number in the cows with ketosis but no difference in the MF cows compared to Control. Phosphoantigen-induced proliferation of γδ T cells has been frequently impaired in patients with haematological malignancies (Wilhelm *et al*., 2003). Impaired phosphoantigen-induced proliferation of peripheral γδ T cells has been reported not only in cancer patients but also in the course of certain chronic infection (Dieli *et al*., 2007). In this study, lower lymphocyte proliferation stimulated with IL-2 was found in Ketosis Group compared to Control Group. IL-2 promotes long term proliferation of activated T cells, signaling through a receptor complex consisting of IL-2 specific IL-2 receptor α (Benczik and Gaffen, 2004). Since, expression of IL-2 receptor on surface of γδ T cell is higher than other T cells (Ohtsuka *et al*., 2011), decreased reactivity of IL-2 might be due to the lower number of γδ T cells in Ketosis Group. Proliferation of bovine PBMC was suppressed under the presence of ketone body *in vitro* (Franklin *et al*., 1991). Although it was not clear whether serum ketone body was high in Ketosis Group, lower numbers of lymphocyte such as γδ T cell might be caused by ketones produced in the body in this group.

E. coli or *S.A.* produces an array of immune evasion factors that target various components of host immune defense. LPS, an outer membrane component of *E. coli*, is a potent activator of monocytes and macrophages (Raetz and Whitfield, 2002). The powerful super-antigen and super-antigen-like molecules of *S.A.* universally bind MHC class II and T cell receptors to induce profound T cell activation (Langley *et al*., 2010). Since significantly lower lymphocyte proliferation and cytokines mRNA expression were found only in PBMC stimulated by *S.A.* in Ketosis Group, it suggests the lower reactivity to *S.A.* stimulation by monocytes or lymphocytes in the cows of this group. Bovine T cells demonstrate cytolytic activity and express IL-2, IL-4, IL-10, IFN-γ and tumor necrosis factor alpha (TNF-α) (Brown *et al*., 1994; Collins *et al*., 1996). Staphylococcal enterotoxins stimulate $CD4^+$ and $CD8^+$ T cell proliferation, but not MHC class-II^+ APC or B cells (Schmaltz *et al*., 1995). Pathogen-associated molecular patterns, such as peptidoglycan and beta-glucan of gram-positive bacteria, can prime bovine γδT cells and exhibit profound proliferation in response to IL-2 in the absence of antigen (Jutila *et al*., 2008). Therefore, the low reactivity of PBMC stimulated by *S.A.* may be due to poor responsiveness of lymphocytes rather than monocytes in Ketosis Group.

In the cows with mastitis caused by Gram-positive or Gram-negative environmental pathogens, there was an increased level of plasma BHB before on set (Jánosi *et al*., 2003). Suppression of IFN-γ stimulated by PHA was affected closely by increased concentration of serum ketone bodies in lactating cows (Kandefer-Szerszen *et al*., 1992). IFN-γ production is regulated in part by IL-2 (McDyer *et al*., 2002), which is required for sustained IFN-γ production (Reem and Yeh, 1984). Therefore increased BHB was suggested to suppress the cellular immune response and to be a one of the risk factor for bovine mastitis (Gregory *et al*., 1993). It was a possible that ketosis decreased T cell numbers and reactivity to bacterial stimulation in the Ketosis cows during the lactation stage.

In this study, significantly lower cytokines expression following lower number PBMC was found only *S.A.* stimulated samples from Ketosis Group. The innate immune system recognizes Gram-positive bacteria mainly through Toll Like Receptor (TLR)-2. Previous study of human monocytes showed that increased TLR-2 expression and ability to detect Gram-positive bacterial peptidoglycan led to significantly higher cytokine production (Dasu *et al*., 2008; Esen *et al*., 2004; Takeda *et al*., 2003). Since bacterial soluble antigen from the wild type of *S.A.* was use dinour present study, PBMC could be stimulated by various antigens. The lower number of monocytes in Ketosis group might have suppressed cytokine productions via TLR-2 signaling in PBMC of this group. However, we do not know whether the reduction in this TLR-2 signaling was the reason for lower expression of cytokines. Further study is needed to clarify this hypothesis.

The present study demonstrated that leukocyte population and cytokine mRNA expression of PBMC stimulated by Gram positive or negative bacteria were different after the onset of two metabolic diseases. Since the causes of immune suppression between hypocalcemia and hyperketonemia are different (Franklin *et al*., 1991; Gregory *et al*., 1993; Kimura *et al*., 2002), the phenomenon and mechanism of immune suppression of these two diseases are also different in the affected cows. It is necessary to clarify the detailed immune function under the presence of Gram-positive or negative bacteria in the cows after the onset of these metabolic diseases.

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