Effect of Body Condition Score of the Dairy Cow on the in vitro Immune Response of Peripheral Blood Mononuclear Cells to Progesterone Stimulation

Hiromichi OHTSUKA1)*, Yurie MURASE1), Takaaki ANDO1), Masayuki KOHIRUIMAKI2), Machiko MUKAI2), Masaaki OIKAWA1), Kiro R PETROVSKI3) and Stephen MORRIS3)

1) School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628, 2) Kohiruimaki Animal Medical Service, Tohoku, Aomori 039–2683, Japan and 3) College of Sciences, Massey University, Palmerston North 4442, New Zealand

(Received 29 July 2008/Accepted 21 November 2008)

ABSTRACT. Immune function in cows is closely associated with their physical and hormonal conditions. In order to clarify the relationship between the body condition score (BCS) of lactating dairy cows and the immune response to progesterone (P4) in vitro, we examined whether lower BCS in dairy cows affects the responsiveness of peripheral blood mononuclear cells (PBMCs) to P4 added in to culture medium. Forty-two non-pregnant healthy Holstein dairy cows were examined at 61 to 120 days after calving. The cows were divided into the following two groups; Low BCS group (N=20), which had a BCS of less than 2.25, and a Control group (N=22), which had a BCS over 2.75. PBMCs were stimulated with P4 (1 μg/mL) and/or phytohemagglutinin (PHA), and the levels of cytokine mRNA were analyzed. In the Low BCS group, a significantly lower IFN-γ level was stimulated by PHA only compared with the Control group. The combination of P4 and PHA significantly decreased the IFN-γ/IL-4 ratio in the Control group, but this reaction was not found in the Low BCS group. Our data indicated that expression of IFN-γ mRNA was basically lower in the low BCS dairy cows and that addition of P4 did not suppress the cellular immune function in these cows. In this study, we observed that P4 reduced the cellular immune response in the adequate BCS cows, whereas immunosuppression by P4 was not found in the PBMCs of the low BCS cows, which already had a lower level of immune function.

KEY WORDS: body condition score, dairy cow, immune function, progesterone.

MATERIALS AND METHODS

Forty-two Holstein dairy cows that were 61 to 120 days after calving and kept at 12 dairy farms were used in this study. All cows were housed in tie stall barns. The presence of a corpus luteum was confirmed in all cows by rectal palpation in order to confirm presence of the estrous cycles (non-pregnant). The peak luteal phase was avoided since the serum P4 concentration during this phase can reach over 1 ng/mL. All cows were confirmed to be clinically healthy on the day that blood samples were collected and remained non-pregnant. No periparturient diseases were observed in the cows used in this experiment. The cows were divided into the following two groups; Low BCS group (N=20), which had a BCS under 2.25 (Scale 0–5) and an average days after calving of 100.6 ± 4.8, and a Control group
Blood sampling was performed for each individual animal at 61 to 120 days post-calving; samples were collected into tubes, which contained no anticoagulant, heparin or dipotassium-EDTA. The serum was separated from the blood in the tubes without the use of an anticoagulant and stored at -30°C until analysis. The serum was used to measure total cholesterol and ketone bodies (enzyme assay) and blood urea nitrogen (urease indophenol method). Hematocrit and total white blood cell counts (WBC) were determined with blood collected into dipotassium-EDTA using a blood cell counter (PC607, ERMA, Germany).

Serum P4 concentration was measured by enzyme immunoassay using anti-progesterone (FK-304E, Cosmo Bio Co., Ltd., Tokyo, Japan) as the 2nd antibody. The P4 assay was performed for all samples at the same time to avoid interassay variations, and the intra-assay coefficient of variation was 4.0%.

Freshly isolated leukocytes were subjected to flow cytometry as described previously [14]. Two ml of blood samples from the tubes containing dipotassium-EDTA were mixed with 4 ml of 0.83% ammonium chloride solution to lyse the red blood cells, and the leukocytes were then separated. WBCs (1 x 106) were incubated with monoclonal antibodies prepared in PBS at 4°C for 60 min. The primary antibodies and descriptions of the working solutions are presented in Table 1. Two-color flow cytometric analysis was performed with fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 antibody, and phycoerythrin (PE)-conjugated Goat-anti-mouse IgG secondary antibody (ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A.) added to PBS for 30 min. Following the second incubation, the samples were twice washed with PBS, and between 10,000 and 20,000 events were collected per condition on a FACScan cytometer (Becton Dickinson, Bedford, MA, U.S.A.); the data were then analyzed using the CellQuest software (Becton Dickinson). The number of each cell population was calculated by multiplication of the percentage by WBC count.

Heparinised blood was used for analysis of cytokine mRNA expression. First, 2 x 106 PBMCs in a total volume of 1 mL of 10% FCS-RPMI were placed in a 24-well plate and stimulated with phytohemagglutinin (PHA; Sigma-Aldrich) for 12 hr at 37°C. After incubation, PBMC pellets were harvested, and the cells were resuspended using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) to collect RNA from the cells. Real-time PCR was used to measure the mRNA levels of cytokines as previously described [14]. Two micrograms of total RNA from each sample were used for synthesis of first-strand cDNA with oligo-dT primers (Invitrogen, Carlsbad, CA, U.S.A.) and Superscript II Reverse Transcripts (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocols. Real-time PCR was performed with SYBR Green Master Mix on an ABI prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.).

Statistical analysis was performed using the Student’s t test for each parameter in the two groups, and the differences between the groups were considered significant at P<0.05. The mean and standard errors of the clinical and laboratory data were calculated.

RESULTS

Table 2 shows the data obtained from the blood analysis. A significantly higher level of β-hydroxy butyrate (BHB) was found in the Low BCS group compared with the Control group. There were no marked differences in any other parameters between the two groups. The serum P4 concentration was higher in the Low BCS group compared with that in the Control group, but there was no significant difference between the 2 groups.

The data for the leukocyte populations are shown in Table 3. The numbers of CD3+ and CD8+ T cells in the Low BCS group tended to be lower (not statistically significant) compared with those in the Control group. Significantly lower numbers of WC1-N3+ T cells were found in...
DISCUSSION

P4 is essential for the establishment and maintenance of pregnancy, including ovulation and uterine and mammary gland development [10]. The major sources of P4 during pregnancy are the corpus luteum of the ovary and, in many species including humans and rodents, the placenta [24]. P4 favors Th2 cytokine production and reduces the number of Th1 cytokines in immune cells [25]. The current study examined whether the effect of P4 on immune cell responses differs between dairy cows with low BCS and those with a BCS of more than 2.75. Th1 to Th2 cytokines in the Control group that manifested as a decrease in the IFN-γ level in isolated PBMCs stimulated with P4, and this was similar to the results of studies with human cells [3]. These results suggested that the PBMCs from dairy cows with adequate BCS were susceptible to P4, but that the PBMCs from the dairy cows with a low BCS were not.

Dairy herds that frequently experience periparturient disease tend to receive a nutritionally poor diet. Depressed cellular immune function, such as low IFN-γ levels and low numbers of CD4+ T cells, have been observed in dairy cows fed a poor diet during the periparturient period [14, 26]. Several metabolic stresses in dairy cows, such as malnutrition and disorder of fat metabolism around the time of calving, can result in lower responsiveness of immune cells to stimulations during the lactation period. Subclinical ketosis is frequently observed during the lactation period in dairy herds with poor diets around the time of calving [8]. In the current study, several subsets of T cell numbers were lower in the Low BCS group. Significantly lower numbers of γδ T cells and total T cells were observed in the Low BCS group. The γδ T lymphocyte can produce high amounts of IFN-γ in response to stimulation with cytokines [17]. Therefore, a decrease in T cell numbers, especially γδ T lymphocyte might affect cytokine production in Low BCS cows. Our results suggest that the low IFN-γ level of the lymphocytes after stimulation with P4 in the Low BCS group might have been due to subclinical ketosis based on the high serum BHB levels in these cows. Ketotic cows tend to have suppressed lymphocyte proliferation because ketone bodies directly suppress lymphocyte blastogenesis stimulated by mitogens in the dairy cow [22]. Therefore, this decreased lymphocyte proliferation is thought to be associated with decreased immune function [2]. Fatty acids play an important role in regulation of immune responses, thus manipulation of dietary lipids affects production of T-cell-derived cytokines in mice [27]. A previous study reported that the increased plasma fatty acid levels in ketosis or starvation might impair immune function by causing changes in lymphocyte metabolism in mitochondria through activation of the caspase cascade [16]. These findings suggested that the lower ability to produce IFN-γ in Low BCS cows might enhance the systemic immunosuppression already elicited

The asterisk indicates a significant difference between the groups (p<0.05 by Student’s t-test). The data expressed as means ± S.E.

| Table 2. Blood parameters and serum progesterone concentrations of the two groups |
|-----------------------------|-----------------------------|-----------------------------|
| Parameter                  | Low BCS group (N=20)        | Control group (N=22)        |
| Ht (%)                     | 30.9±0.6                   | 33.1±0.9                   |
| WBC (× 10^3/μl)            | 83.3 ± 4.4                 | 89.6 ± 5.0                 |
| T-cho (mg/dl)              | 220.0±9.2                  | 220.5±11.2                 |
| BUN (mg/dl)                | 14.8±0.7                   | 13.9±0.5                   |
| BHB (μmol/l)               | 774.5±62.4                 | 567.6±39.6*                |
| P4 (ng/ml)                 | 3.25±0.66                  | 1.90±0.50                  |

These data were analyzed for each individual non-pregnant cow. The asterisk indicates a significant difference between the groups (p<0.05 by Student’s t-test). The data expressed as means ± S.E.

| Table 3. Peripheral leukocyte populations of the two groups |
|-----------------------------|-----------------------------|-----------------------------|
| Cell number                 | Low BCS group (N=20)        | Control group (N=22)        |
| CD3+ (× 10^3/μl)            | 15.0±1.5                    | 18.0±2.3                    |
| CD4+ (× 10^3/μl)            | 5.8±0.7                     | 8.0±1.2                     |
| CD8+ (× 10^3/μl)            | 3.0±0.5                     | 3.5±0.5                     |
| CD4+/CD8+                   | 2.52±0.36                   | 2.34±0.20                   |
| WC1-N3+ (× 10^3/μl)         | 2.0±0.3                     | 2.9±0.4*                    |
| MHC class-II’CD14+ (× 10^3/μl) | 11.4±1.0                  | 10.6±1.0                    |
| MHC class-II’CD14+ (× 10^3/μl) | 4.2±0.4                    | 3.8±0.4                     |
| MHC class-II’CD14+ (× 10^3/μl) | 2.5±0.5                     | 4.0±0.06                    |

These data were analyzed for each individual non-pregnant cow. The asterisk indicates a significant difference between the groups (p<0.05 by Student’s t-test). The data expressed as means ± S.E.
by subclinical ketosis. As P4 suppressed the production of IFN-γ in the PBMCs of the normal BCS cows, the reduced responsiveness to the suppressive effect of P4 in the Low BCS cows might be due to already existing systemic immunosuppression.

The target T cell of P4 was not clarified in the present study. P4 directly suppresses production of IFN-γ by naive CD4+ T cells in the mouse [13]. In contrast, P4 inhibits the effector function of activated cytotoxic T cells in the mouse [12]. Scheibl and Zerbe [23] suggested that progesterone suppresses specific components of the immune system and natural killer (NK) cell activity in cows. These reports suggest that P4 might interfere with several T cells or NK cell activation. As BHB induces inhibitory effects on the proliferation of bovine bone marrow cells in in vitro culture [7], the lymphocyte numbers of the Low BCS cows with higher BHB levels were expected to be lower. The lower responsiveness to P4 in the Low BCS cows might be due to a lower number of circulating lymphocytes, including the target lymphocytes of P4, in the Low BCS group.

In the present study, we also observed that P4 is one of the hormones that suppresses to cellular immune function in dairy cows, but we did not observe this effect in thin cows. The IFN-γ expression was hardly suppressed by P4 in the Low BCS cows due to their already existing low levels of IFN-γ expression responsiveness. However, it is still unclear whether or not P4 had no effective to immune response in the low BCS cows. P4 containing medicines are frequently administered in treatment of repeat-breeding cows because high milk yield dairy cows have low progesterone levels during the luteal phase; however the immunosuppressive effect of this steroid hormone should be considered when determining the appropriate treatment [1]. Further studies are needed to clarify the mechanism of the effect of P4 on lymphocyte function in dairy cows in order to maintain their health and prevent mastitis.

ACKNOWLEDGEMENTS. Authors are indebted to all experimental farm owners and farm personnel in Aomori Prefecture for their understanding and cooperation.

REFERENCES


Fig. 1. Cytokine mRNA expressions in PBMCs from the lactating cows of the 2 groups. A. Levels of IL-1α mRNA. PBMCs were stimulated with either PHA alone (□) or a combination of PHA and P4 (◆). B. Levels of IL-4 mRNA. PBMCs were stimulated with either PHA alone (□) or a combination of PHA and P4 treated PBMCs (◆). C. Levels of IFN-γ mRNA. PBMCs were stimulated with either PHA alone (□) or a combination of PHA and P4 treated PBMCs (◆). D. Ratio of IFN-γ/IL-4 for either PHA alone (□) or a combination of PHA and P4 treated PBMCs (◆). The IFN-γ/IL-4 ratio was calculated as (IFN-γ mRNA expression)/IL-4 mRNA expression). The Low BCS and Control groups contained 20 and 22 cows, respectively. Cytokine mRNA expression was analyzed using heparinised blood. First, 2 x 10⁶ PBMCs were stimulated with PHA for 12 hr at 37°C. The asterisk indicates a significant difference between the cultures containing PHA alone and the combination of PHA and P4 (P<0.05 by Student’s t-test). The different letters indicate significant differences between the 2 groups (P=0.05 by Student’s t-test). Data are expressed as means ± S.E.


