FULL PAPER *Internal Medicine*

Changes in Peripheral Blood Mononuclear Cells after Calving in Lactating Cows with Serious Decrease of Body Weight before and after Calving

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ABSTRACT. The aim of this study was to clarify the hormonal and immunological changes in periparturient dairy cows with excessive weight loss following calving. We analyzed leukocyte populations and the peripheral blood mononuclear cell (PBMC) mRNA of IFN-γ, IL-4, growth hormone (GH)-receptor (R) and insulin -R and measured the GH and insulin concentrations. Ten dairy cows fed in one herd were used in this study and were divided into two groups. Five cows that experienced loss of body weight following calving of over 10% comprised the Weight Loss Group, and the remaining five cows, which experienced loss of body weight of 5% or less following calving, served as the Control Group. The Weight Loss Group had high serum cholesterol and low blood urea nitrogen levels compared with the Control Group throughout the period of observation. In regard to the leukocyte populations, there were significantly fewer $CD4^+CD45R$-T cells in the Weight Loss Group 1 to 2 months after calving compared with the Control Group. The percentage of IFN-γ/IL-4 among PBMCs in the Weight Loss Group tended to be lower than in the Control Group throughout the observation period. In the Weight Loss Group, the levels of GH-R during month 2 were lower than in the Control Group. We concluded that excessive loss of body weight in dairy cows following calving is associated with depressed cellular immune function.
KEY WORDS: body weight, dairy cow, immune function, periparturient.

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Dairy cows starting lactation in a thin condition lack adequate energy reserves and peak at a lower milk yield. Experience and research have shown that cows gaining weight (in positive energy balance) at the time of insemination have a higher conception rate than cows losing weight. Cows with lower dry matter index (DMI) develop greater body weight (BW) loss during early lactation. Changes in body weight are closely related to metabolism of several hormones. Because cows that are underfed mobilize body reserves of fat or protein, they have higher plasma concentrations of growth hormone (GH), NEFA and BHBA and lower concentrations of glucose, insulin (INS) and leptin around calving [23]. Furthermore, several hormones, acting through their receptors, are essential for somatic growth and development and for maintaining metabolic homeostasis, including the immune system [14, 17].

Dairy cows are most susceptible to infections at or around the time of calving. We previously observed that reduction of body weight in cows before and after calving is closely related to the occurrence of periparturient diseases, including mastitis and retained placenta in dairy cows [13]. Their immune response is usually inadequate to combat the stress of calving, and their appetite is insufficient to meet the demands of early lactation. Previous research has shown that cows with mastitis or retained placenta have suppressed immune function prior to calving [20].

Body weight is positively associated with somatic cell score in lactating dairy cattle [3]. Recent investigation suggests that dairy cows with the lowest dry matter intake during the dry period had the most cellular immune suppression after calving [21]. Therefore, we hypothesized that immune suppression and endocrine disorders would be observed in dairy cows with excessive loss of body weight following calving. The aim of this study was to clarify the changes in immunological and endocrine conditions in dairy cows with excessive loss of body weight following calving.

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MATERIALS AND METHODS

Cows: We chose ten clinically healthy dairy cows in one herd for use in this study. There was no occurrence of disease in the ten cows during observation. They were divided into two groups; five cows whose body weight (BW) decreased by over 10% after calving comprised the Weight Loss Group (N=5), and five cows whose body weight loss after calving was 5% or less comprised the Control Group (N=5). Average age and interval between calving of the Weight Loss and Control Groups were 3.3 ± 0.2 and 376.8 ± 18.2 days and 3.6 ± 0.4 and 396.4 ± 19.6 days, respectively. BW and body condition score (BCS) were measured about 2 weeks before (pre-calving) and after calving, and measurements of BW and BCS and collection of blood samples were performed monthly after calving. Body weight was measured by estimation tape as previously reported [13].

Blood analysis: Blood samples were collected into tubes containing either no anticoagulant, heparin or dipotassium-EDTA between 3 and 5 p.m. before feeding. Serum samples were used to measure serum total cholesterol (TC; enzyme assay), blood urea nitrogen (BUN; urease indophenol

method) and nonesterified fatty acids (NEFA; enzyme assay). Total white blood cell counts (WBC) were determined with a blood cell counter.

Hormone Assay: The concentrations of plasma growth hormone and insulin were measured by time-resolved immunofluorometric assay as previous reported [24]. The intra-assay coefficients of variation were 4.1% and 9.3%, respectively.

Flow cytometry: We mixed 2 m*l* of blood samples with 4 m*l* of 0.83% ammonium chloride solution and separated the leukocytes. All leukocyte samples were finally resuspended in cold PBS, and 1×10^6 cells were incubated with one of the primary monoclonal antibodies for 1 hr. The lineage-specific monoclonal antibodies (VMRD, Pullman, WA, U.S.A.) used were CACT183A or CACT83B (T-helper lymphocyte, CD4 antigen), BAT82A or BAQ111A (T-cytotoxic/suppressing lymphocyte, CD8 antigen) and GC6A (non-activated leukocyte, CD45R antigen), CAT82A (monocyte/B lymphocyte, MHC class II antigen) and MY-4 (monocyte, CD14 antigen; Coulter Immunology, Hialeah, Florida, U.S.A.). Two-color flow cytometric analysis was performed. The cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) -labeled secondary antibody (Cappel, Durham, NC, U.S.A.) at 4°C for 30 min, and then analyzed with Lysis II software by FACScan (BD Biosciences Immunocytometry Systems, San Jose, CA). Data from 10000 events per sample were analyzed using software (Cell Quest; Becton Dickinson).

Real-time PCR: The real-time PCR method has been previously described in detail [16, 21]. We placed 10^6 PBMC in 1 m*l* of 10%FCS-RPMI medium into a 24-well plate, and stimulated the cells with phytohemagglutinin (PHA, Sigma, 5 μg/m*l*) for 12 hr at 37°C. The cells were then resuspended using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.)

Two micrograms of total RNA from each sample were used for synthesis to 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 using SYBR Green Master Mix on an ABI prism 7,700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.). The target DNA sequences were specifically amplified with the primers as previously designed from bovine IL-4 (forward 5' TGCCCCAAAGAACACAACTG 3'; reverse 5' TTTAGCCTTTCCAAGAGGT C3'), bovine IFN-γ (forward 5' AGCCCAGATGTAG CTAAGGG3'; reverse 5' CTCCAGTTTCTCAGAGC TGC3'), bovine INS-receptor (R) (forward 5' ATGGAGGAGTCTGCTGGCGAATG C3'; reverse 5' GGCACTGA CATAGGCAGCCACGCT3') and bovine GH-R (forward 5' TTATGCCCAGGTAAGCGACATTAC3'; reverse 5' TGATTTTGTTCAGTTGGTCTGTGC3').

The comparative threshold cycle number ($2^{-\Delta\Delta Ct}$) method was used after a validation experiment demonstrated that the target and reference efficiencies (β-actin) were approximately equal. The Ct values define the threshold cycle of PCR, at which amplified products were detected. Fold changes in expression for the two groups (ΔΔCt) were calculated from the arithmetic formula $2^{-\Delta\Delta Ct}$ as described previously [16].

Statistical Analysis: Statistical comparisons between the 2 groups were made using Student's *t*-test assuming equal variance, and values of $p<0.05$ were regarded as significant. The mean values and standard errors of the clinical and laboratory data were calculated.

RESULTS

BCS decreased after calving in both groups, but the difference was not significant. Body weight fell substantially in the Weight Loss Group during month 1, but increased immediately thereafter. In the Control Group, body weight decreased gradually after the pre-calving measurement and recovered during month 3. Significant differences in body weight were observed from month 1 to 2 between the 2 groups. The milk yield of the Weight Loss Group was higher than that of the Control Group during month 1, but no significant differences were observed (Fig. 1).

The blood biochemical data are shown in Fig. 2. TC levels remained higher in the Weight Loss Group compared with those in the Control Group throughout the observation period, and these differences were significant from after

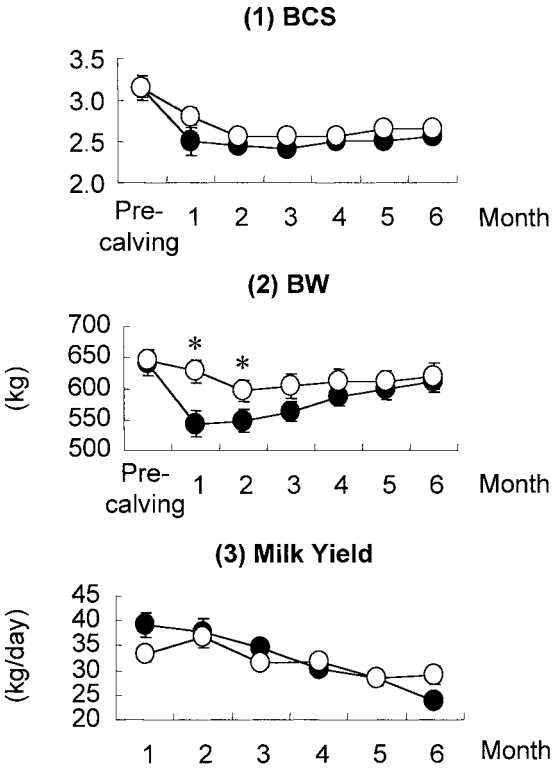

Fig. 1. Changes in the BCS (1), BW (2) and milk yield (3) of the Weight Loss (●) and Control Groups (○). Values are expressed as means ± SE. Different letters indicate significant differences between the 2 groups ($P<0.05$).

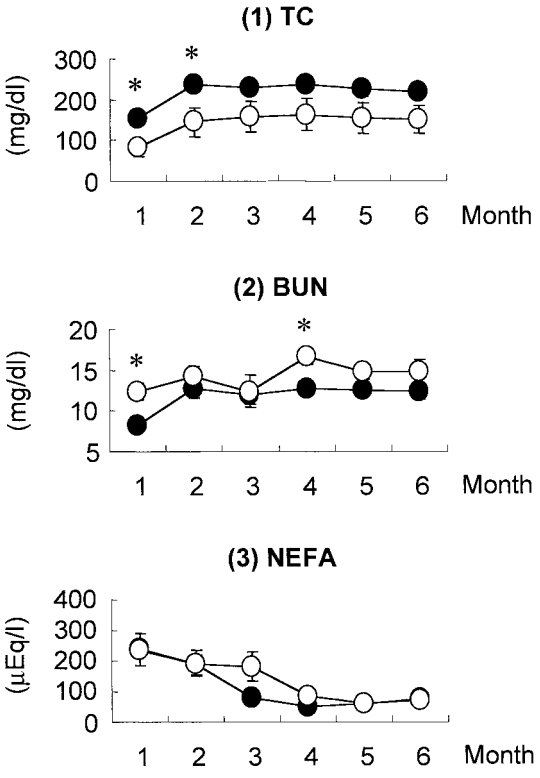

Fig. 2. Changes in the TC (1), BUN (2) and NEFA (3) of the Weight Loss (●) and Control Groups (○). For further comments, see the legend to Fig. 1.

calving to month 2. BUN levels were significantly lower in the Weight Loss Group during month 1 and month 4 compared with the Control Group. There were no significant differences in NEFA levels between the two groups.

The numbers of $CD4^+CD45\ R^+$ T cells and $CD4^+CD45\ R^-$ T cells in the Weight Loss Group tended to be low between months 1 and 3, and a significantly high number of $CD4^+CD45\ R^-$ T cells was observed during months 1 and 2. The numbers of these T cells were stable in the Control Group during our observations (Fig. 3). No significant differences were found in the numbers of MHC class ll or CD14 positive cells between two groups during this study (data not shown).

The $CD4^+/CD8^+$ ratio of the Control Group was highest during month 1, and this difference was significant. However, this ratio was stable in both groups after month 2. The ratios of IFN-γ/IL-4 remained lower in the Weight Loss Group compared with the Control Group throughout this experiment, although the differences were not significant (Fig. 4).

The changes in hormone concentrations and expression of hormone receptor RNA levels are shown in Fig. 5. There was no difference in GH concentration between the two groups. The INS concentration of the Weight Loss Group was higher than that of the Control Group between months 1 and 4, and a significant difference was observed during

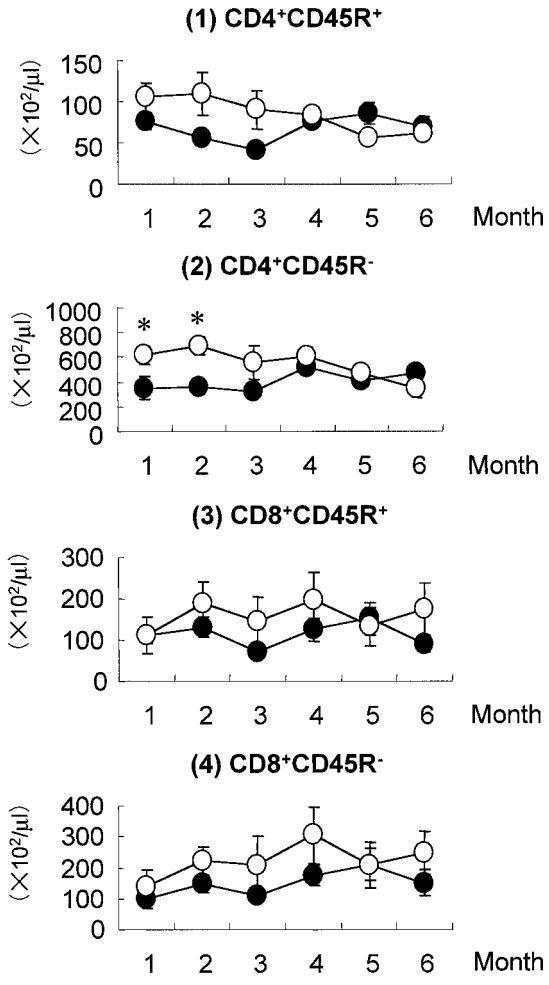

Fig. 3. Changes in the numbers of $CD4^+CD45R^+$ (1) $CD4^+CD45R^-$ (2) $CD8^+CD45R^+$ (3) and $CD8^+CD45R^-$ (4) cells of the Weight Loss (●) and Control Groups (○). For further comments, see the legend to Fig. 1.

month 3. The GH-R level was low in both groups during month 1 and peaked during month 3. The GH-R level of the Weight Loss Group was significantly lower than that of the Control Group during month 2. In the Weight Loss Group, INS-R was reduced between months 5 and 6 and was significantly lower than that of the Control Group during month 5.

DISCUSSION

Immunosuppression occurs in most dairy cows around calving. However, dairy cows with infectious diseases often experience a variety of immune-suppressive factors, such as an unsuitable nutritional program, stressful environment conditions and negative energy balance (NEB) around calving, and immunosuppression in such cows is the result of interactions among these factors. In general, mastitis is more frequent in dairy cows with lowered BCS or BW during early milking periods [25]. It has been hypothesized that

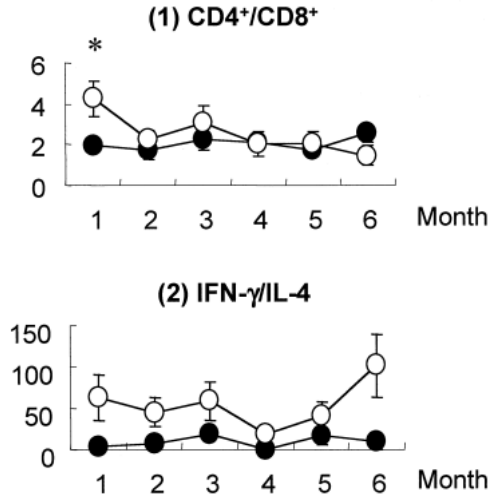

Fig. 4. Changes in the $CD4^+/CD8^+$ (1) and IFN-γ/IL-4 rates (2) of the Weight Loss (●) and Control Groups (○). For further comments, see the legend to Fig. 1.

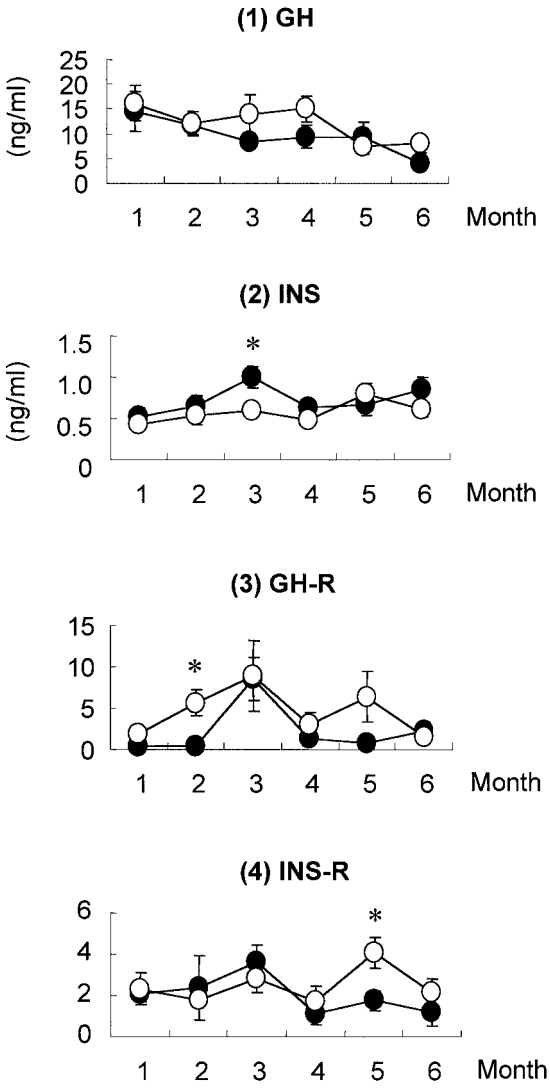

Fig. 5. Changes in the serum GH concentration (1), serum INS concentration (2), GH-R (3) and INS-R (4) of the Weight Loss (●) and Control Groups (○). For further comments, see the legend to Fig. 1.

the occurrence of mastitis is related to immunosuppression in dairy cows following decline of BCS or BW during the periparturient period [3].

This study indicated that immunosuppression occurred in the dairy cows with excessive loss in BW after calving. Previous investigation [9] suggests that pre- or periparturient changes in energy balance, such as higher plasma concentrations of ketone body or NEFA, reduce immune cell function, and these are important factors related to occurrence of infectious diseases in cows at the first lactation. However, our present results indicated higher serum TC and non-elevated NEFA in the cows with excessive loss of BW. Serum cholesterol levels in lactating cows have been recognized to be related with milk yield or DMI [11], and a higher milk yield and serum cholesterol level were observed in the Weight Loss Group after calving. Loss of weight in these cows after calving seemed to be due to high milk production ability because the accumulated nutrition was used for production of milk in the lactating cows. Moreover, lower levels of BUN were notable in the Weight Loss Group during month 1 compared with the Control Group. It is possible that the cows in the Weight Loss Group were hypoproteinemia and that this affected milk production rather than negative energy balance. We previously observed that the $CD3^+$T cell counts was low in herds where mastitis appears frequently during milking periods following declines in BUN [19]. Low BUN in lactating cows indicates a protein energy deficiency [4]. Such a protein deficiency might have reduced the $CD4^+$T cell count or IFN-γ/IL-4 ratios of the Weight Loss Group because protein energy deficiency induces depression of cellular immune function [6]. We suspect that in addition to NEB, protein deficiency is also an immune-suppressive factor in periparturient dairy cows.

Appearance of lower $CD4^+$T cell counts or lower $CD4^+/CD8^+$ and IFN-γ/IL-4 ratios in the peripheral lymphocytes of cows is known to indicate an immune-suppressive condition [12, 22]. IFN-γ, which is produced by T cells, is a central cytokine in the development of cellular immune responses [27]. In this study, $CD4^+CD45R^-$ T cell counts and IFN-γ/IL-4 ratios remained lower in the Weight Loss Group until month 2 after calving. $CD4^+CD45R^-$ T cells are activated helper T cells because CD45R is expressed on non-activated T cells, and when activated, T cells lose CD45R [1]. Naive $CD4^+$ T cells produce IL-2, but not IL-4 or IFN-γ, while the Memory $CD4^+$ T cells produce mRNA for IL-2, IL-4 and IFN-γ after stimulation with mitogen [2]. This study confirmed that the dairy cows experiencing excessive loss of body weight around calving were at

increased risk of infection during the early lactating periods due to suppression of T cells.

In dairy cows, increased circulating GH and decreased circulating insulin during early lactation are well known to promote the mobilization of adipose tissue stores needed to supplement dietary energy consumption [26]. NEB is known to be a key risk factor that causes increased incidence of metabolic disease during the periparturient and early postpartum periods. NEB induces large decreases in INS concentrations and increases in GH resulting in substantially lower BCS after calving [23]. Therefore, increased GH and decreased insulin during early lactation are usually observed in dairy cows, and our results showed a similar pattern of hormonal changes during month 2. The insulin concentration increased temporarily in the Weight Loss Group during month 3. Amino acids and glucose act synergistically to increase plasma insulin release in lactating dairy cows [15]. In this study, the two group of cows were fed using the same feeding conditions, it might be influence to increased amino acid or glucose by the increase of individual DMI in Weight Loss Group rather than changes in quantity or contents of the feeding. However, the cause of the changes in insulin was not clarified.

Previous study has demonstrated that GH and INS stimulate the proliferation or production of cytokines in activated T cells [7, 22]. GH-R or INS-R are expressed on peripheral leukocytes, and these cells are both targets for GH or INS stimulation [8, 10]. These data provide a molecular basis to study the factors controlling GH-R or INS-R expression and regulation of immune function [5]. The lower GH-R expression of the Weight Loss Group during month 2 might have been due to depression of lymphocyte response stimulated by PHA. Previous report have found that periparturient Holstein cows frequently experience immunosuppression following reduction of glucose transport activity in T cells [18]. We doubtful that GH played a role in the immune system of the periparturient dairy cows preventing the occurrence of infectious disease. However, the effect of these two hormones against immune cells and the role of their hormones receptors in activation of lymphocytes were not clear in the cows with serious body weight loss.

This study indicated that the change in BW around calving was closely related to immune system function in the dairy cows. The cause of loss of BW in periparturient dairy cows is not simple, and may be due to such factors as depression of appetite, poor quality feed, poor feeding program and drastically raised milk production. Complicated metabolites may be involved in mobilizing body protein or energy to compensate for insufficient nutrients in cows with excessive loss of BW. Further studies are necessary to define the roles of various hormones in the host immune system, as well as the contributions of different metabolites around the calving period.

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