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—Original—

Immune Responses during the Peripartum Period in Dairy Cows with Postpartum Endometritis

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Abstract. Determining the immune responses to the development of endometritis during the peripartum period may assist in the development of more efficient reproductive management regimens for dairy herds. In this study, we compared the peripartum immune responses of dairy cows that develop endometritis by 4 weeks postpartum (n=11) to cows that did not develop this disease (n=19). Blood samples were collected 1 week before calving, just after or during calving, and then at weeks 1, 2, 3, and 4 postpartum. Cows that developed endometriris had significantly higher total leukocyte, neutrophil, lymphocyte, and monocyte counts than the control cows ($P<0.05$) at all time points. The leukocytes from cows that developed endometritis were significantly less phagocytic than those from control cows at all sampling time points ($P<0.01$). The serum TNFα concentrations of the control cows decreased linearly from the prepartum time point ($P=0.0029$), but the endometritis cows showed a different profile ($P>0.05$). As a result, the serum TNFα concentrations were greater in the endometritis group ($P<0.01$) than in the control group during the third and fourth weeks postpartum. The greater total leukocyte numbers and neutrophil, lymphocyte and monocyte counts, and the maintenance of elevated serum TNFα levels in the cows with endometritis may be due to infection in the postpartum period. Furthermore, the decreased phagocytic capacity of leukocytes during the peripartum period, including at the prepartum time point, makes cows more susceptible to postpartum endometritis.

Key words: Dairy cows, Endometritis, Leukocytes, Phagocytic capacity, TNFα

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Endometritis is a common reproductive disease in cattle that causes economic loss [1, 2]. Uterine contamination following calving is common, but most healthy cows are able to clear the uterus of bacteria within the first 2 to 3 weeks after calving [3]. However, cows that cannot eliminate the infection may subsequently develop endometritis [4]. Various risk factors, such as calving problems, a retained placenta, metabolic disorders, and the parity or nutritional status of the cow have been related to the development of postpartum endometritis, although some of them are controversial [5–13]. Since many dairy cows experience sudden nutritional and endocrine changes during the peripartum period [14, 15], this may lead to aberrant immune function that could predispose the cows to severe uterine infections [16]. The inflammatory and immune response to uterine infection compromises the animal's welfare and causes subfertility and infertility [17]. Understanding the mechanisms underlying the effect of uterine bacterial contamination and its associated immune response may assist in the development of more efficient treatment regimens and prevention protocols.

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After pathogen attack, immune cells detect bacterial components such as endotoxin and peptidoglycan *via* toll-like receptors, which activate downstream signaling to stimulate the release of cytokines, including TNFα and various interleukins (IL-1, IL-6, and IL-8) [18]. These cytokines provide a positive feedback loop to further increase immune cell mobilization [17]. Neutrophils are the most important phagocytic cells to be recruited from the peripheral circulation to the uterine lumen. However, the functional capacity of neutrophils is reduced after parturition in many cattle [19]. This decreased neutrophil activity during the peripartum period has been suggested to influence the occurrence of endometritis [20, 21]. Another study has found that both phagocytic activity and bactericidal activity may be reduced in cows with experimental endometritis [22]. However, these studies were mainly conducted in normal puerperium cows [20, 23, 24], in cows with induced endometritis [22], or *in vitro* [25]. Only a few studies examining naturally occurring endometritis during the peripartum period in dairy cows and its associated immune response have been published [21, 26]. Determining the immune responses to the development of endometritis during the peripartum period may assist in the development of more efficient reproductive management regimens for dairy herds. Therefore, we compared the immune responses of dairy cows with or without postpartum endometritis during the peripartum period. To do this, we analyzed the number of peripheral blood leukocytes, their phagocytic capacity, and the serum TNFα level.

Materials and Methods

Animals

This study was performed during the period of April to August 2003 on two Holstein dairy farms located in Chungbuk province in central Korea. Holstein cows were maintained in free-stall facilities, fed a total mixed ration, and milked twice daily. Thirty pregnant Holstein cows, with a range of 1 to 3 lactations (2.0 ± 0.2), were used for this study.

Blood sampling

Blood samples were collected from the tail vein of each cow to analyze the total and differential leukocyte counts (neutrophils, eosinophils, lymphocytes, and monocytes), the phagocytic capacity of the leukocytes, and the serum TNFα level. The samples were taken 1 week before calving (prepartum day 6.2 ± 0.4), just after or during calving (postpartum day 0.1 ± 0.4), and then at weeks 1, 2, 3, and 4 postpartum. Two ml of the blood samples were placed into 5 ml plastic tubes coated with EDTA-2K for the analysis of total and differential leukocyte counts. Ten ml of the blood samples were placed into plastic centrifuge tubes coated with heparin for evaluation of the phagocytic capacity of the leukocytes. An additional 10 ml of the blood samples were placed into plastic centrifuge tubes without additives for the analysis of serum TNFα levels. All blood samples were immediately placed in an ice bath after collection. The total and differential leukocyte counts and the isolation of leukocytes in the blood samples were performed within 1 h after blood collection. The blood samples used to evaluate TNFα were centrifuged (2,500 g for 20 min at 4 C), and the sera were frozen at –20 C until use.

Diagnosis of endometritis

The presence of endometritis was assessed at 4 weeks postpartum by the corresponding author and diagnosed as positive if a cloudy discharge and an enlarged uterus, as observed by rectal examination, were observed with or without other clinical signs. Therefore, the endometritis group may also have included cases of metritis and pyometra [8, 27]. Cows were thus categorized into the endometritis group (n=11) or the control group (n=19).

Total leukocyte numbers and counts of differential leukocytes

The total numbers of peripheral blood leukocytes and the counts of differential leukocytes were measured using an automatic blood cell counter (Hemavet 850, Drew Scientific Inc., Dallas, TX, USA).

Leukocyte isolation

To prepare leukocytes, the blood samples were centrifuged at 200 g for 30 min at room temperature. The buffy coat was collected, washed three times with phosphate-buffered saline (PBS) at pH 7.6, resuspended with Tris-HCl buffer (pH 7.6)

containing 0.83% NH_4Cl and 1% bovine serum albumin, and incubated at 37 C for 5 min to remove contaminating red blood cells. The resulting cells were resuspended to the desired concentration in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 0.02 mg/ml gentamicin, and 5% fetal bovine serum (FBS; Gibco). The viability of the resulting leukocytes, as determined by a trypan blue dye exclusion test, was always more than 98%.

Evaluation of phagocytic capacity

The phagocytic capacity of the leukocytes was determined as described previously [28]. Thus, leukocytes at a density of 5×10^6 cells/ml were placed into each well of a 24 well-plate (cell culture plate, flat bottom, 21.5 cm^2; Nunc A/S, Roskilde, Denmark). Subsequently, 20 μl of 5×10^9 particles/ml of fluorescein isothiocyanate (FITC)-labelled latex beads (size: 2.0 μm; Polysciences, Inc., Warrington, PA, USA) were added to the leukocytes, and the plates were incubated for 2 h at 37 C under a 5% CO_2 humidified atmosphere. The leukocytes were then harvested gently by slow pipetting, centrifuged at 400 g for 4 min, and washed 3 times with PBS containing 3 mM EDTA-2Na. Leukocytes that had phagocytized the latex beads (per 10,000 leukocytes) were then immediately counted by flow cytometry (FACS Calibur, BD Biosciences- Immunocytometry Systems, Franklin Lakes, NJ, USA). The results were expressed as a percentage of absolute phagocytic capacity.

Determination of serum TNFα level

The serum TNFα levels were determined by a cytotoxicity assay using the TNFα- sensitive murine fibrosarcoma WEHI 164 cell line (American Type Culture Collection, Manassas VA, USA) as described previously [29]. Briefly, WEHI 164 cells were suspended at a concentration of 2×10^5 cells/ml in DMEM medium (Sigma-Aldrich Co., St. Louis, MO, USA) containing 10% FBS, 0.4 mg/ml streptomycin, and 0.4 mg/ml penicillin, and then transferred to 96-well cell culture microplates (flat bottom, 0.33 cm^2; Nunc A/S) in 100 μl/well aliquots. The individual sera were diluted 1:40 in DMEM medium containing 10% FBS, and added as 100 μl /well aliquots. The assay included a standard, namely, varying concentrations of human recombinant TNFα (Antigenix America Inc., Huntington, NY, USA). The plates were incubated at 37 C for 48 h, after which 20 μl 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4 disulfophenyl) -2H-tetrazolium, monosodium salt (WST-1) (Dojindo Lab., Kumamoto, Japan) solution was added to each well. The plates were then further incubated for 1 h at 37 C to allow color development. The plates were read at 450 nm using an automated microplate reader (EL × 808, Bio-tek® Instruments, Inc., Winooski, VT, USA). The amounts of TNFα in the test sera were calculated on the basis of the standard curve obtained in the same assay. In this assay, absorbance is inversely correlated with cell death or lysis due to exposure of WEHI-164 cells to TNFα.

Statistical analysis

Results are given as means ± standard error (SEM). Statistical analyses were performed by using the SAS software [30]. Repeated-measures ANOVA was used to compare the changes in mean total and differential leukocyte counts, the phagocytic capacity of the blood leukocytes, and the serum TNFα levels between the endometritis and control groups. The statistical model for repeated-measures ANOVA included the effects of group, the sampling day, and the interaction between group and sampling day (group × sampling day). If a significant interaction occurred between a group and sampling day with regard to a given parameter (i.e. the different groups showed distinct response profiles over time), the effect of sampling day was evaluated within each group and differences between the sampling days between groups were analyzed using one-way ANOVA. A probability level of $P<0.05$ was considered significant.

Results

The changes in mean total and differential blood leukocyte count for the cows in the control and endometritis groups are shown in Fig. 1. No significant interaction between group and sampling days occurred in any of the leukocyte count parameters ($P>0.05$). The endometritis cows differed from the control cows in that their total leukocyte, neutrophil, lymphocyte, and monocyte counts were greater at all sampling time points ($P<0.05$). For both groups, compared to the 1 week

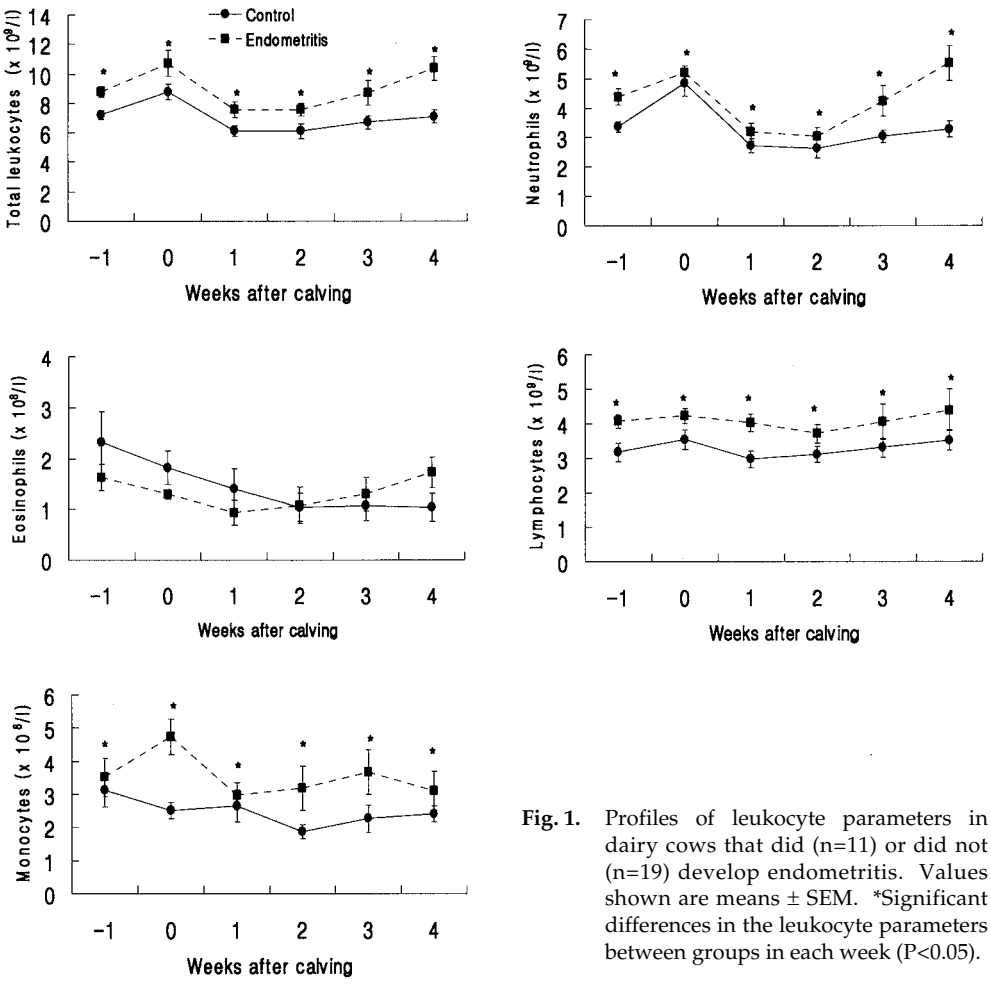

Fig. 1. Profiles of leukocyte parameters in dairy cows that did (n=11) or did not (n=19) develop endometritis. Values shown are means ± SEM. *Significant differences in the leukocyte parameters between groups in each week (P<0.05).

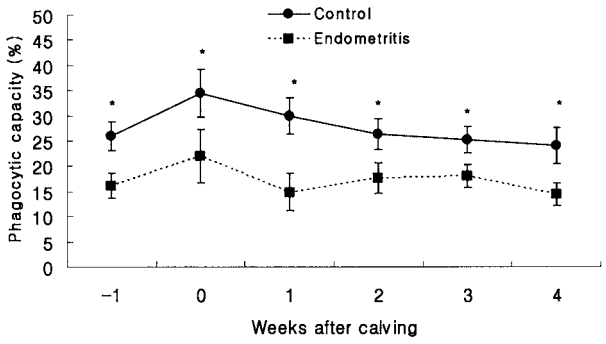

Fig. 2. Changes in the mean phagocytic capacity of dairy cows that did (n=11) or did not (n=19) develop endometritis. Values shown are means ± SEM. *Significant differences in the phagocytic capacity between groups in each week (P<0.01).

prepartum counts, the total leukocyte, neutrophil, and lymphocyte counts had increased by the calving period; this elevation then dropped during the first week postpartum. Thereafter, the counts increased gradually over the remainder of the study.

Figure 2 shows the leukocyte phagocytic capacity of the two cow groups and how this capacity changed over the sampling days. No significant interaction between groups and sampling days occurred in the phagocytic capacity of leukocytes (P>0.05). The phagocytic capacity of leukocytes was uniformly greater (P<0.01) in the control group than in the endometritis group. For both groups, the leukocyte phagocytic capacity increased during calving relative to the week 1 prepartum values, but then dropped by the first week postpartum to approximately the start values. This level was then maintained for up to 4 weeks postpartum.

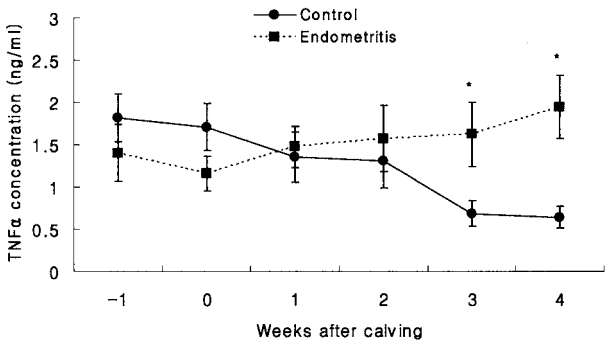

Fig. 3. Changes in the mean serum TNFα concentrations in dairy cows that did (n=11) or did not (n=19) develop endometritis. Values shown are means ± SEM. *Significant differences in the serum TNFα concentrations between groups in each week (P<0.01).

The changes over time in the mean serum TNFα concentration of the control and the endometritis cows are shown in Fig. 3. A significant interaction did occur between group and sampling day (P= 0.002) for the serum TNFα concentrations. The serum TNFα concentrations in the control group decreased linearly (P=0.0029) from week 1 prepartum to 4 weeks postpartum, while those in the endometritis group did not (P=0.69). As a result, the serum TNFα concentrations were lower (P<0.01) in the control group than in the endometritis group during the third and fourth weeks postpartum.

Discussion

To determine the relationship between the development of postpartum endometritis by dairy cows and their peripheral blood leukocyte immune responses, the total and differential leukocyte numbers, the phagocytic capacity of the leukocytes, and the serum TNFα levels were compared for cows that did or did not develop endometritis in the peripartum period. Our results reveal that the development of postpartum endometritis is related to increased total and differential leukocyte counts and increased serum TNFα levels, along with a reduced phagocytic capacity.

For both the cows that did and did not develop endometritis, calving was associated with an increase in the total leukocyte, neutrophil, and lymphocyte counts relative to the week 1 prepartum counts. These counts dropped by the first week postpartum, but then tended to increase again over the following three weeks. This general profile for total and differential leukocyte counts is similar to that reported by Mateus *et al*. [26] and Hussain and Daniel [31]. Prepartum leukocytosis is mediated by an antepartum rise in cortisol levels [32], while the decrease in the first week postpartum is associated with the migration of the leukocytes towards the uterine lumen and mammary gland [33]. We found that cows that developed endometritis had greater total leukocyte, lymphocyte, and monocyte counts than the control group. Similar results have been reported by Cai *et al*. [21]. However, while we found neutrophil counts were also higher in cows that developed endometritis, Cai *et al*. reported that these cows had lower neutrophil counts than cows without endometritis after parturition. They speculated that this decrease in circulating neutrophil counts may be related to elevated neutrophil migration after parturition. However, Kehrli *et al*. [20] have shown that the ability of neutrophils to migrate decreases markedly after parturition. Thus, it remains unclear whether the circulating neutrophil counts in cows that develop endometritis are lower or higher than in control cows. An important observation was that the total leukocyte, lymphocyte, and neutrophil counts in cows that go on to develop endometritis are already higher before calving than in control cows, even though these values remains within the normal physiological ranges. Since calving is a time at which the uterus becomes infected with bacterial pathogens, the prepartum differences between the cow groups may suggest that the cows may already have been stressed in some way other than infection. We speculated that this pre-existing condition weakens the cows and predisposes them to being unable to fight off uterine pathogens upon calving.

To accomplish their host defense roles, neutrophils perform a series of activities. The function of neutrophils in host defense mechanisms relies heavily on phagocytosis. Previous studies [20, 33, 34] showed that polymorphonuclear cell (PMN) function was impaired in the cow during the peripartum period, which suggested that attenuated PMN function contributed to the increased incidence of postpartum diseases. We found that for both the endometritis and control

cow groups, the phagocytic capacity of their leukocytes increased at calving relative to the week 1 prepartum capacity, and that this then dropped within a week to the start values; these levels were then maintained for the remainder of the study. Newbould [35] reported an increase in phagocytosis before parturition that peaked at week 2 prepartum and then rapidly decreased after parturition. Similarly, Kehrli *et al*. [20] found high ingestion activity during the peripartum period and a slight decrease after parturition. Significantly, we found that the leukocytes of cows that develop endometritis showed a lower phagocytic capacity than those of the control cows, and that this difference was already present before calving. This is consistent with the notion that the endometritis associated with parturition may be an immunosuppressive disease [16]. Thus, decreased phagocytic capacity during the prepartum period may be a factor that predisposes cows to developing postpartum endometritis.

Previous studies have revealed that many different cytokines are responsible for inflammatory disorders and the activation of immune cells [36–39]. One of these cytokines is TNFα, which induces many gene products involved in inflammation, tissue repair, hematopoiesis, immune response, and anti-tumor effects [40]. TNFα is produced in response to invasive stimuli, such as bacterial, viral, fungal, parasitic, or neoplastic agents, through several signal pathways [41]. Conversely, it is also believed to be an inflammatory mediator of acute and chronic infection, and over-production of TNFα appears to be involved in a number of pathological conditions, including septicemia and cachexia [42–44]. In this study, the serum TNFα concentrations in the control group decreased linearly from week 1 prepartum to 4 weeks postpartum, while those in the endometritis group did not. As a result, the serum TNFα concentrations were higher in cows with endometritis during the third and fourth weeks postpartum compared with those without endometritis. These data suggest that persistent bacterial infection in the uterus maintains the higher serum TNFα levels, while the control cows clear their uterus of bacterial contamination, and as a result, their TNFα levels drop. The results of the cows without endometritis also suggest that the immune function system is activated during the peripartum period, and that the immune system recovers from pregnancy around the third week postpartum.

In conclusion, we suggest that the higher total leukocyte numbers, higher neutrophil, lymphocyte, and monocyte counts, and the sustained elevations in serum TNFα levels in the cows with endometritis may be due to infection in the postpartum period. Moreover, we showed that impaired neutrophil function before parturition and during postpartum may predispose cows to the development of postpartum endometritis.

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