Changes of immunophysiological characteristics in neonatal calves experimentally challenged with mixture of live bacteria and virus

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ABSTRACT

The aim of the present study was to define efficient immunophysiological parameters in neonatal Holstein calves with an experimentally induced microbial infection. Calves ($n = 15$) were challenged with classical swine fever virus (LOM strain) and *Erysipelothrix insidiosa* live vaccine by intravenous injection at 3 wk of age except for control calves ($n = 4$). The level of total serum IgA was significantly increased at 14 and 19 d post-experimental challenge (DPEC) compared with that in calves at -2 DPEC. At 5 DPEC, relative amounts of bacterial- and viral-specific IgA increased significantly and were sustained until 26 DPEC. In the hematology assay, the neutrophil:lymphocyte ratio (%) in whole blood was significantly decreased at 14 DPEC because of a significant increase in lymphocytes and a coincident decrease in neutrophils. The percentages of CD4⁺ and CD25⁺ T cells were significantly decreased at 14 DPEC and returned to initial levels at 19 DPEC. It is intriguing to note that the level of serum lactoferrin was significantly decreased by the microbial challenge within 1 d. The concentration of haptoglobin was increased within 3 d and gradually decreased in calves after microbial challenge. Our results suggest that 1) bovine serum lactoferrin plays an important role in the innate immune response against microbial infection at an early stage and 2) experimentally induced microbial challenge using porcine live bacterial and viral vaccine in calves could be a good experimental model to evaluate the effect of diet or stress induced by environmental change on the immune responses against microbial infection.

Key words: neonatal calf, experimental model, lactoferrin, microbial infection

INTRODUCTION

Neonatal calves have heightened susceptibility to a variety of infectious diseases because of the developmental immaturity of their immune systems together with high stress conditions including weaning and diet changes. High morbidity and mortality of neonatal calves due to microbial infection cause substantial economic loss to producers. Therefore, a strategy to enhance immune responses in neonatal calves is required. In the present study, we attempted to provide an appropriate biomarker and an experimental model to evaluate immune responses. It has been suggested that finding a biomarker of the immune status in a calf herd could be a useful tool for health management in the field (Charina et al., 2006). Recently, many researchers have focused on the discovery of appropriate biomarkers of microbial infection to aid in early detection, diagnosis, and therapy (Jacobsen et al., 2004; Doherty et al., 2007). Cytokines and other mediators of inflammation are known to play roles in the innate immune response (Bannerman, 2009). Acute phase protein (APP) is stimulated by inflammatory mediators and involved in the initial reaction against infection, inflammation, trauma, or stress. The function of APP is to bind harmful molecules and debris produced upon tissue damage and to promote the elimination of pathogenic organisms and tissue repair, preventing further injury (Chan et al., 2004). Previous studies have examined the effect of experimentally induced microbial infection on the concentration of iron binding protein and APP (McNair et al., 1998; Heegaard et al., 2000; Grell et al., 2004). Lactoferrin (Lf) is an iron-binding glycoprotein present in various secretions (e.g., milk, tears, saliva, serum). It is also stored in specific granules of polymorphonuclear granulocytes from which it is released after microbial activation. Lactoferrin is known to exert bactericidal activity as well as immunoregulatory functions upon microbial challenge by modulating IL-1, IL-2, and tumor-necrosis factor- α , and enhancing monocyte and natural killer cell cytotoxicity (Caccavo et al., 2002). Although the effect of Lf as an immune enhancer has

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been well studied, the Lf level in the blood circulation and its relevance during microbial infection has not been assessed in cattle, to the best of our knowledge. Haptoglobin (**Hp**), a hemoglobin-binding protein, is considered a representative APP in cattle and known to be a useful indicator of bacterial infection: the concentration of haptoglobin increases from 100- to 1,000-fold within 24 h of inflammation in cattle (Conner et al., 1998). However, its mechanism of action is not fully understood.

The objectives of the present study were to characterize a profile of physiological parameters in relation to peripheral immune responses of neonatal calves with experimentally induced microbial infection to find appropriate biomarkers for indication of microbial infection, and to evaluate the proficiency of an experimental infection model in calves using porcine live vaccine. The hypothesis of the current study was that experimentally induced microbial infection would alter the activity of immunoregulatory cells and their products in neonatal calves.

MATERIALS AND METHODS

Animals, Management, and Diet

The experiments were carried out at the Dairy Science Division, National Institute of Animal Science, South Korea. All procedures were reviewed and approved by the ethics committee on the use of animals in research, National Livestock Research Institute, South Korea. Holstein calves ($n = 15$; 8 male and 7 female, BW = 37.06 ± 2.62 kg) born during September 2007 were separated from their mothers within 2 h of birth, weighed, and moved into individual pens (1.5×2.5 m; bedded with wood shavings) where they were fed colostrum at 10% of BW for the first 3 d. Pens had solid iron-rod sides with an opening in the front and rear to allow calves free access to calf starter, chopped mixed grass hay, and water from a bowl drinker in each pen. Calf starter and forage were given from the first and fourth weeks of age, respectively. All calves were fed whole milk stored in a milk tank at approximately 4 to 6°C after milking, by using mobile plastic bottles (2-L capacity) fitted with soft rubber nipples. Calves were fed using a step-down milking method (Khan et al., 2007). The milk was provided at a rate of 20% of BW until 28 d old, gradually reduced to 10% at 29 to 30 d old, and that level was maintained for the remaining 14 d of the weaning period. All calves were weaned at 6 wk of age (21 d post-experimental challenge; **DPEC**). Additional Holstein calves ($n = 4$; 1 male and 3 female, BW = 32.14 ± 3.41 kg) were assigned as a negative control group and received no experimental challenge.

Health Scoring

The scoring system used to monitor the health of calves was as follows. For respiratory scoring, normal = 1; slight cough = 2; moderate cough = 3; moderate to severe cough = 4; severe and chronic cough = 5. Diarrhea scoring was determined by fecal fluidity, consistency, and odor, and was conducted daily (0800 h) using the procedure of Larson et al. (1977). Diarrhea scoring was performed as follows. For fecal fluidity, normal = 1; soft = 2; runny = 3; watery = 4. For fecal consistency, normal = 1; foamy = 2; mucoid = 3; sticky = 4; constipated = 5. For fecal odor, normal = 1; slightly offensive = 2; highly offensive = 3. When diarrhea score exceeded 3 (average of fluidity, consistency, and odor for 2 consecutive days) or when a calf exhibited other signs of disease (fever or cough), antibiotic therapy (sulfadimethoxine sodium, 55 mg/kg of BW daily; Green Cross Veterinary Products Co. Ltd., Yongin, South Korea) was initiated, and the therapy was continued until visual signs of disease disappeared (maximum of 5 d). Scours were treated with electrolyte therapy (Eltradd, 3 g/L in drinking water; Bayer Animal Health Co., Suwon, South Korea).

Experimental Infection and Blood Collection

To investigate the immune response against microbial infection, all calves ($n = 15$) except those in the negative control group were challenged with classical swine fever virus (LOM strain) and *Erysipelothrix insidiosa* live vaccine (1 mL, Green Cross Veterinary Products Co. Ltd.) by intramuscular injection at 3 wk old (0 DPEC).

For hematological, flow cytometry, and serum antibody analyses, blood samples were collected from the jugular vein at 19 (–2 DPEC), 26 (5 DPEC), 35 (14 DPEC), and 40 (19 DPEC) d of age. For the lactoferrin and haptoglobin ELISA, additional blood samples (10 mL) were collected into evacuated tubes coated with lithium-heparin (Vacutainer System, Becton Dickinson, Brondby, Denmark) at 22 (1 DPEC), 24 (3 DPEC), 43 (22 DPEC), 45 (24 DPEC), and 47 (26 DPEC) d of age.

Hematology

Plasma was harvested from anticoagulated blood after centrifugation at $1,600 \times g$ at 4°C for 15 min and stored at –80°C until the assays were conducted. Neutrophil, lymphocyte, platelet, monocyte, and leukocyte populations in whole blood were measured with an automatic analyzer (Hemavet 850, Drew Scientific, Sylva, NC).

Quantification of Lf by ELISA

The amounts of Lf in plasma were determined using commercial ELISA kits based on Lf binding capacity (Bethyl Laboratories, Montgomery, TX). In brief, the bovine Lf capture antibody was coated in each well of 96-well immunoplates (Nalgene Nunc International, Rochester, NY) and incubated for 1 h. The plates were then washed 3 times with washing buffer (0.05% Tween 20 in PBS) and blocked with blocking buffer (1% BSA in PBS) for 30 min. After washing, the plates were incubated with bovine serum and respective standard proteins for 1 h. The detection antibody conjugated with biotin was added to the plates after washing and incubated for 1 h. The specific binding was detected by treating with streptavidin–horseradish peroxidase (HRP) followed by tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO). The reaction was stopped with 2 *N* H₂SO₄, and absorbance was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Serum Hp (Life Diagnostics Inc., West Chester, PA) and total serum immunoglobulin concentrations (Bethyl Laboratories) were measured using an ELISA assay kit according to the procedure of the manufacturer. Levels of antigen-specific immunoglobulin in the serum samples were determined by indirect ELISA. Hog cholera and *Erysipelothrix insidiosus* live vaccine dissolved in PBS (1 mL) was centrifuged at 13,000 × *g* for 1 min, and the supernatant was harvested and transferred into a new tube for the collection of virus. Then, the pellet was dissolved in PBS (1 mL) for the isolation of bacteria. Bacterial antigens were sonicated (Vibracell, Sonic & Materials Inc., Danbury, CT) and centrifuged at 50,000 × *g* for 2 h at 4°C, and protein concentration was measured by Bradford assay (Bradford, 1976). Each well of the microtiter plates was coated with bacterial (10 µg/mL) or viral antigen (1:100 dilution). Serum antigen-specific IgG and IgA concentrations were measured using a bovine IgG and IgA ELISA assay kit (Bethyl Laboratories) according to the procedure of the manufacturer. In brief, bacterial and viral antigens were coated in 96-well immunoplates (Nalgene Nunc International) and incubated overnight at 4°C. The plates were then washed with washing buffer (0.05% Tween 20 in PBS) 3 times and blocked with washing buffer (0.05% Tween 20 in PBS) for 2 h. After washing, the plates were incubated with diluted bovine serum for 3 h at room temperature. The anti-bovine IgG (or IgA) HRP-conjugated antibody was added to the plates after washing and incubated for 2 h. The specific binding was detected using streptavidin-HRP and TMB substrate (Sigma-Aldrich). The reaction was stopped with 2 *N* H₂SO₄. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices).

Quantification of CD4⁺, CD8⁺, and CD25⁺ Cells

Single-color flow cytometric analysis was performed as follows: 5 × 10⁵ cells were harvested and stained with CD4-APC, CD8-APC, or CD25-PE (VMRD, Pullman, WA). After staining for 30 min at 37°C, the cells were washed 3 times with PBS, and differences in the expression of cell surface molecules were detected using a flow cytometer FACScanto (BD Biosciences, San Jose, CA). All data files were further analyzed with FACSDiva software (BD Biosciences).

Statistical Analysis

Data were analyzed using PROC MIXED (SAS Institute Inc., Cary, NC) with calves as the experimental unit and time relative to experimentally induced microbial infection as fixed effects. Data from negative control animals were analyzed separately. Data are presented as means ± standard errors of the means, and the associated *P*-values were derived from the statistical analysis of appropriately transformed data using the model described above. The overall effects of time relative to experimentally induced microbial infection were considered significant when *P* < 0.05.

RESULTS

Clinical Observation and Feed Intake

Clinical response and feed intake of calves during experimental period is presented in Table 1. During the experimental period, 2 calves were treated with antibiotic therapy because of severe scouring and they were removed from the experiment. Calf starter intake in the experimental animal was gradually increased until the end of the experimental period. Microbial challenge did not influence health score (incidence of scour and respiratory disease) although those scores generally decreased with age. Microbial infection challenge also did not affect intake of milk and calf starter (35.79 vs. 35.08 L, 0.56 vs. 0.86 kg). Lower milk and higher calf starter intakes during 7 to 13 DPEC and 14 to 20 DPEC compared with −14 to 6 DPEC were due to the stepdown milk feeding program employed in this experiment.

Hematology

Hematology analysis, as influenced by microbial infection, is shown in Table 2. Percentage of neutrophils declined after microbial infection with the most significant (*P* < 0.05) influence on 19 DPEC. Microbial infection significantly increased (*P* < 0.05) circulating percentage of lymphocytes until 19 DPEC. Therefore,

Table 1. Intake of milk and calf starter (CS) and fecal and health scores (mean $\pm$ SE) in calves after experimental challenge

DPEC ¹	Milk intake ² (L)	CS intake (kg)	Fecal score ³	Health score ⁴
-14 to -6	34.18 $\pm$ 1.50 ^a	0.24 $\pm$ 0.08 ^c	4.00 $\pm$ 0.25 ^a	5.12 $\pm$ 0.45 ^a
-7 to -1	35.79 $\pm$ 1.10 ^a	0.56 $\pm$ 0.06 ^c	3.33 $\pm$ 0.14 ^a	4.52 $\pm$ 0.23 ^{ab}
0 to 6	35.08 $\pm$ 2.20 ^a	0.86 $\pm$ 0.14 ^{bc}	4.00 $\pm$ 0.32 ^a	4.39 $\pm$ 0.67 ^b
7 to 13	27.53 $\pm$ 0.38 ^b	1.37 $\pm$ 0.12 ^b	2.13 $\pm$ 0.19 ^b	4.01 $\pm$ 0.49 ^b
14 to 20	20.26 $\pm$ 0.20 ^c	3.07 $\pm$ 0.47 ^a	3.20 $\pm$ 0.10 ^a	3.89 $\pm$ 0.32 ^b

^{a-c}Means within same column with different superscripts differ ($P < 0.05$).

¹DPEC = days post-experimental challenge (0 DPEC = day of experimental challenge).

²Milk intake: the calves were given colostrum and milk for 28 d at the rate of 20% of BW, which was reduced to 10% of BW for the remaining period and all calves were weaned at 21 DPEC (stepdown method).

³Fecal score = diarrhea score $\times$ scouring days.

⁴Health score = average of fecal score and respiratory score.

the neutrophil:lymphocyte (N:L) ratio (%) was significantly decreased at 14 and 19 DPEC (0.71 ± 0.09 and $0.65 \pm 0.08\%$, respectively; $P < 0.05$) compared with -2 DPEC, probably because of higher lymphocytes and lower neutrophils after microbial infection. The concentration of leukocytes in calves significantly ($P < 0.05$) declined at 19 DPEC from that at -2 DPEC ($9.21 \pm 0.67 \times 10^9$ vs. $8.08 \pm 0.52 \times 10^9$ cells/L). A tendency for decreasing platelets and thrombocytopenia was observed in calves after experimental challenge with microbes. The platelet concentration significantly ($P < 0.05$) declined at 14 and, 19 DPEC when compared with -2 DPEC. The reduction of neutrophils, N:L, and platelets, and the elevation of lymphocytes after experimental infection were not observed in control animals, except for a significant ($P < 0.05$) reduction of platelets at 19 DPEC.

Changes of Total and Antigen-Specific Immunoglobulins

Levels of both total serum and antigen-specific IgG in the serum against bacterial and viral antigens were not

changed in calves after microbial challenge (Figure 1A, Figure 2A and 2B). As shown in Figure 1B, however, total serum IgA concentration was significantly ($P < 0.05$) elevated at 14 and 19 DPEC compared with -2 DPEC (78.78 and 83.53 vs. 40.92 μ g/mL, respectively) in calves with experimental infection. Although control calves showed a tendency for elevation of total serum IgA in a time-dependent manner, this was of a lower magnitude compared with that in the experimentally infected model.

A significant ($P < 0.05$) increase in viral (hog cholera) and bacterial (*Erysipelothrix insidiosus*) specific serum IgA was also observed in calves with experimental microbial challenge (Figure 2A and B). The concentration of bacterial and viral specific IgA reached the highest level at 5 DPEC followed by gradual decrease, although the values remained at a higher ($P < 0.05$) level than at -2 DPEC.

Changes in T Cells

Microbial challenge significantly ($P < 0.01$) decreased CD4⁺ T cells at 14 DPEC (Figure 3A). The

Table 2. Changes of leukocytes and platelet counts (mean $\pm$ SE) in calves with experimental infection

DPEC ¹ /group	Neutrophils (N, %)	Lymphocytes (L, %)	N:L (%)	Leukocytes (10^9 cells/L)	Platelets (10^9 cells/L)
-2					
Challenge	42.94 $\pm$ 1.9 ^a	49.20 $\pm$ 1.5 ^b	0.90 $\pm$ 0.07 ^a	9.21 $\pm$ 0.6 ^a	558.46 $\pm$ 26.7 ^a
Control	40.56 $\pm$ 4.3 ^a	51.13 $\pm$ 3.2 ^a	0.79 $\pm$ 0.1 ^a	6.82 $\pm$ 0.8 ^a	459.12 $\pm$ 26.8 ^a
5					
Challenge	39.62 $\pm$ 2.3 ^a	53.68 $\pm$ 2.2 ^a	0.78 $\pm$ 0.07 ^a	8.31 $\pm$ 0.5 ^a	492.44 $\pm$ 47.9 ^a
Control	41.73 $\pm$ 3.6 ^a	47.62 $\pm$ 4.7 ^a	0.87 $\pm$ 0.1 ^a	8.24 $\pm$ 1.5 ^a	504.28 $\pm$ 54.8 ^a
14					
Challenge	37.11 $\pm$ 2.6 ^a	56.57 $\pm$ 2.5 ^a	0.71 $\pm$ 0.09 ^b	8.21 $\pm$ 0.7 ^a	462.17 $\pm$ 39.6 ^b
Control	42.28 $\pm$ 5.1 ^a	49.10 $\pm$ 2.4 ^a	0.86 $\pm$ 0.1 ^a	6.52 $\pm$ 0.8 ^a	494.53 $\pm$ 51.4 ^a
19					
Challenge	34.09 $\pm$ 2.4 ^b	58.92 $\pm$ 2.1 ^a	0.65 $\pm$ 0.08 ^b	8.08 $\pm$ 0.5 ^b	469.11 $\pm$ 29.5 ^b
Control	48.58 $\pm$ 5.4 ^a	46.09 $\pm$ 3.6 ^a	1.05 $\pm$ 0.1 ^a	7.94 $\pm$ 0.7 ^a	390.30 $\pm$ 28.4 ^b

^{a,b}Means within the same column in each group with different superscripts differ significantly ($P < 0.05$).

¹DPEC = days post-experimental challenge (0 DPEC = day of experimental challenge).

percentage of CD25⁺ T cells was also significantly ($P < 0.01$) decreased at 14 DPEC ($2.15 \pm 0.60\%$) compared with -2 DPEC ($4.89 \pm 1.36\%$; Figure 3C). The ratios of CD4⁺:CD25⁺ and CD8⁺:CD25⁺ significantly ($P < 0.05$) increased at 14 DPEC (Figure 4B and C), primarily because of a significant decrease in CD25⁺ cells in calves with experimental infection. In control animals, the ratio of CD4⁺:CD25⁺ significantly ($P < 0.05$) increased at 5 DPEC because of a decrease in CD25⁺ cells (Figure 4B).

Lf Levels

Serum Lf was significantly decreased ($P < 0.001$) within a day by microbial challenge (Figure 5A). The concentration of Lf decreased from 454.47 ± 39.32 ng/mL at resting stage (-2 DPEC) to 256.33 ± 32.99 ng/mL at 1 DPEC and then the level was maintained for 5 d. Among 15 calves challenged with microbes, 14 calves responded to microbial infection with reduced Lf. However, this significant reduction was not observed in control calves (Figure 5B).

Although there was large variation (454.47 ± 39.32 ng/mL) in Lf concentration among calves at -2 DPEC, microbial infection reduced the Lf level by an average of 41% at 1 DPEC (data not shown). The maximum and minimum Lf reduction was 77.56 and 30.36%, respectively, among calves experimentally challenged with microbes. It is interesting to note that the reduction of milk intake in accordance with the stepdown milk feeding program also induced a reduction of serum Lf level in all calves tested (Figure 5A and B). As shown in Figure 5 and Table 1, serum Lf levels were significantly ($P < 0.05$) decreased at 14 and 19 DPEC when milk intake was 20.26 ± 0.20 L compared with -2 DPEC when milk intake was 35.79 ± 1.10 L. When milk was restricted at 21 DPEC, the average level of Lf decreased significantly ($P < 0.01$) at 22, 24, and 26 DPEC (postweaning) compared with 19 DPEC (preweaning). The Lf concentration was significantly decreased in 11 out of 15 calves after milk restriction treatment. The reduction of Lf by milk restriction ranged from 69.92 to 4.83% (data not shown).

Hp Levels

A level of Hp >50 ng/mL was observed in 4 out of 15 calves after microbial challenge within 3 d (Figure 6A). The average concentration of Hp in calves with microbial challenge tended to increase within 1 to 3 DPEC and the peak response was seen at 3 DPEC. After microbial challenge, the maximum response of Hp concentration was observed at 3 DPEC, and a tendency to return to normal level was found. Microbial challenge

Figure 1. Change of total immunoglobulins in serum from calves with experimental challenge: A) concentration of total serum IgG (mg/mL) and B) concentration of total IgA (μ g/mL). Inf indicates a group with experimental challenge and Con indicates control group; DPEC = days post-experimental challenge (0 DPEC = day of experimental challenge). Means with different symbols differ significantly compared with -2 DPEC (* $P < 0.05$; ** $P < 0.01$).

increased average Hp level by 7.91 to 7,335% during 1 to 5 DPEC, respectively (data not shown). However, individual calves differed significantly with respect to their serum level of Hp after microbial challenge (Figure 6 A). The peak of haptoglobin was not observed in control animals during 1 to 5 DPEC (Figure 6B).

DISCUSSION

The purpose of the present study was to examine the changes of circulating immunophysiological parameters in calves experimentally challenged with microbes. Through these observations, we hoped to identify valid and effective biomarkers for microbial infection, which would greatly help to manage animal health and study immune responses in calves. Blood is known to contain potential biomarkers, because it encompasses an enormous range of physiological processes in the body at any given time (Anderson and Anderson, 2002; Gins-

burg and Haga, 2006). Therefore, in the current study, changes of leukocytes, acute phase proteins, and iron-binding proteins were investigated in the circulation of neonatal calves subjected to microbial infection.

In the present study, porcine live vaccine was used as an experimental model. The results showed that the model using porcine live vaccine successfully induced effective B-cell immune responses in which the total serum IgA concentration gradually increased in a time-dependent manner in calves experimentally infected with microbes. Importantly, the amount of antigen-specific IgA was significantly increased compared with prechallenge infection. Furthermore, not only percentage changes in leukocytes and platelets but also the abrupt response of acute phase proteins such as Lf and Hp were observed in the present experimental model. Although it is possible that the change of immunophysiological parameters was affected by natural aging of the animal or maturation of the immune system, our study showed that control animals did not show such responses. Therefore, it seems reasonable to conclude that neonatal calves were well sensitized to porcine live vaccine when we consider the following facts: 1) significant elevation of antigen-specific IgA occurred after experimental challenge; 2) significantly decreased Lf level was observed within 1 d; 3) although it is obvious, no symptom of microbial infection was observed in control animals.

It is noteworthy that using porcine live vaccine as antigens for experimental infection in a bovine experiment is rare; however, it has an advantage over conventional vaccine because it excludes the possibility of the colostral-derived antibody response from a vaccinated mother (Husband and Lascelles, 1995). It is also important to note that although the current model may not be able to cover all infectious challenges, porcine live vaccine could serve as a suitable model system to study B-cell responses, hematology, and profiles of immune-related serum proteins after microbial infection in calves without severe disease symptoms regardless of colostral status. Moreover, this experimental model can be utilized in evaluating the effect of dietary additives on immune activities against microbial infection.

In the present study, microbial challenge induced changes of Hp and Lf levels. Although the average concentration of Hp significantly increased in calves experimentally challenged with microbes (1 to 3 DPEC), there was great individual variation. Therefore, it is not clear from the present study whether Hp could serve as a reliable biomarker for infection or vaccination. It is well known that LPS from gram-negative bacteria is responsible for high induction of acute-phase inflammatory responses compared with the component in gram-positive bacteria (Boosman et al., 1989; Werling et al.,

Figure 2. Change of antigen-specific immunoglobulins in serum from calves with experimental challenge: relative concentration of A) viral-specific IgA and IgG and B) bacterial-specific IgA and IgG. Inf indicates a group with experimental challenge and Con indicates control group; DPEC = days post-experimental challenge (0 DPEC = day of experimental challenge). Means with different symbols differ significantly compared with -2 DPEC (* $P < 0.05$; *** $P < 0.001$).

1996). It is probable that the gram-positive bacteria used in the present study were not as effective as LPS in inducing a significant increase in acute-phase response. Thus, when APP are considered as biomarkers for the immune response induced by microbial infection, not only Hp (as was used in present study) but also other APP such as serum amyloid A or fibrinogen need to be examined simultaneously in future studies..

Interestingly in the present study, the level of Lf was dramatically ($P < 0.001$) reduced within a day after microbial infection. Furthermore, Lf reduction was observed in 14 out of 15 calves challenged with microbes, unlike the individual variation in Hp responses. It is known that Lf plays a role in a variety of immune responses by enhancing bactericidal activity and immunoregulatory functions such as modulation of cytokine secretion and activity of monocytes and natural killer cells (Caccavo et al., 2002). However, the biological role of Lf is not completely elucidated. Previous study has reported that a bacterial infection caused inflammatory response and altered the level of the iron binding

Figure 3. Change in the phenotypic composition of peripheral blood lymphocytes in calves with experimental challenge: Percentage of A) CD4⁺, B) CD8⁺, and C) CD 25⁺ T cells. Inf indicates a group with experimental challenge and Con indicates control group; DPEC = days post-experimental challenge (0 DPEC = day of experimental challenge). Means with different symbols differ significantly compared with -2 DPEC (*** $P < 0.001$).

protein transferrin (Ogunnariwo and Schryvers, 1990). Although Lf is known to be involved in various immune responses, few attempts have been made to profile the change of serum lactoferrin after microbial infection.

In the current study, not only did serum Lf concentration tend to be decreased with reduction of milk intake in accordance with the stepdown milk feeding system but reduction of Lf level was observed in calves by milk restriction treatment within 1 d. Therefore, it is possible that milk intake potentially affects serum Lf level. However, it is unlikely when we consider that the amount of milk intake was not different pre- and

Figure 4. Change in the ratios of peripheral blood lymphocytes in calves with experimental challenge: A) CD4:CD8; B) CD4:CD25; and C) CD8:CD25 ratio. Inf indicates a group with experimental challenge and Con indicates control group; DPEC = days post-experimental challenge (0 DPEC = day of experimental challenge). Means with different symbol differ significantly compared with -2 DPEC (* $P < 0.05$).

post-challenge (35.79 ± 1.10 L vs. 35.08 ± 2.20 L). For this reason, we are confident that Lf in the milk was not a major factor in the significant reduction in serum Lf after microbial infection. The reduction of Lf in the present study by microbial infection could be explained by the fact that neutrophils containing Lf granules or circulating serum Lf were migrating into the initial site of infection to participate in an immune response within days of infection or vaccination. Such migration should induce the reduction of circulating serum Lf level. It

Figure 5. Change of bovine serum lactoferrin (Lf) in calves with experimental challenge: A) Lf concentration (ng/mL) in calves with experimental challenge and B) change of Lf concentration in control calves ($\circ$ = level of individual calf, $\blacksquare$ = average level of calves). DPEC = days post-experimental challenge (0 DPEC: day of experimental challenge). Means with different symbols differ significantly compared with -2 DPEC (* P < 0.05; ** P < 0.01; *** P < 0.001).

has been shown that Lf has a bacteriostatic property because of its iron-sequestering properties at the local infection site (Lonnerdal and Iyer, 1995).

The percentages of $CD4^+$, $CD8^+$, and $CD25^+$ cells were investigated during the experimental period. A tendency for percentage decreases in $CD4^+$ and $CD8^+$ cells was observed after microbial infection. However, this phenomenon of a significant decrease in the $CD4^+$ T-cell subset and a moderate decrease in $CD8^+$ T cells was similar to the changes in these T-cell subsets in noninfected, control neonatal calves observed by others (Nonnecke et al., 2003; Foote et al., 2007). Therefore, the changes in T-cell populations can be explained by age-related rather than microbial infection-related phenomena. A previous study showed that, within days of infection, the percentage of the circulating $WC1^+$ $\gamma\delta$

T-cell population decreased. Subsequently, the $WC1^+$ $\gamma\delta$ T-cell population was observed to expand in the circulation, suggesting an active response to infection (Pollock and Welsh, 2002). In the present study, reduction of circulating $CD4^+$ and $CD25^+$ cell populations was observed but expansion of the T cell population was not found. Mean proportions of $CD4^+$, $CD8^+$, and $CD25^+$ cells were 16.15 ± 0.74 , 7.07 ± 0.21 , and $3.98 \pm 0.30\%$, respectively, during the 6 wk of the experimental period. Similar levels of $CD4^+$, $CD8^+$, and $CD25^+$ cells in calves have been reported by others (Wilson et al., 1996; Kulberg et al., 2004).

CONCLUSIONS

We conclude that the experimental model using porcine live vaccine in calves would alter concentra-

Figure 6. Change of bovine serum haptoglobin (Hp) level in calves with experimental challenge: A) Hp concentration (ng/mL) in calves with experimental challenge and B) change of Hp concentration in control calves (○ = level of individual calf, ■ = average level of calves); DPEC = days post-experimental challenge (0 DPEC = day of experimental challenge).

tions of Lf, Hp, and immunoglobulins and percentage of leukocytes. It is certain that the present model system using porcine bacteria and virus would not be representative of all bacterial and viral infections in calves, but it could serve as a tool to evaluate the effect of diet and stress under environmental changes on immune responses against microbial infection. Therefore, the present experimental model system could be helpful for studying immune responses in calves, especially in designing calf feeds with capacity to increase resistance to microbial infection. Based on this model, it is suggested that bovine serum Lf may play an important role in the innate immune response against microbes during the early stage of infection.

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