FULL PAPER *Internal Medicine*

Changes in Peripheral Leukocyte Subsets in Dairy Cows with Inflammatory Diseases after Calving

Hiromichi OHTSUKA[1)], Masateru KOIWA[2)], Shigeo FUKUDA[5)], Yohei SATOH[6)], Tomohito HAYASHI[4)], Fumio HOSHI[1)], Tomo-o YOSHINO[3)] and Sei-ichi KAWAMURA[1)]

[1)]Department of Veterinary Internal Medicine, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034–8628, [2)]Veterinary Teaching Hospital, [3)]Department of Veterinary Pathology, Rakuno Gakuen University, Ebetsu, Hokkaido 069–8501, [4)]Science University of Tokyo, Research Institute for Biological Sciences, Chiba 278–0022, [5)]Hokkaido Animal Research Center, Shintoku, Hokkaido 081–0038 and [6)]Department of Nemuro Agricultural Mutual Relief Association, Shibetu, Hokkaido 086–1451, Japan

(Received 1 December 2003/Accepted 18 March 2004)

ABSTRACT. To clarify the cellular immune system in dairy cows with inflammatory diseases after calving, the leukocyte subsets were examined in Holstein dairy cows. Twenty Holstein cows reared in one herd, were used in this study. Nine cows (Group 1) experie nced onset of mastitis or puerperal fever within 2 weeks after calving, and the other eleven cows remained healthy (Group 2) after calving. The numbers of $CD3^+$, $CD4^+$ and $CD8^+$ cells tended to be lower in Group 1 than in Group 2 from the day of calving through week 1. These results suggested that the cows with inflammatory diseases might have experienced a decline in T cells by the day of calving, before the onset of disease.

KEY WORDS: calving, dairy cow, leukocyte subset, periparturient inflammatory disease.

J. Vet. Med. Sci. 66(8): 905–909, 2004

Periparturient inflammatory diseases (PID), such as mastitis or puerperal fever cause an economic damage for a stock raiser. Many cases of PID occur within the first 2 weeks after calving [21]. It has been suggested that depression of leukocyte function during the periparturient period contributes to the susceptibility to infectious diseases in dairy cows, but the leading factors causing immune suppression have not been fully investigated.

Changes in leukocyte subsets have been implicated in local and systemic cellular immune responses. Above all, immunological mechanisms induced by T cells play important roles in host defense [3]. In healthy cows, there is a decrease in $CD3^+$, $CD4^+$ and $CD8^+$ T cells around the calving day, and this is accompanied by physiological changes [8]. This means that calving cows have a higher risk of infectious diseases during the peripartum period. Although van Kampen [20] described the change in lymphocyte subsets in some cows with infectious diseases during the periparturient term, it has not yet been clarified whether marked change occurs in cows with PID after calving. Therefore, the purpose of this study was to examine the peripheral leukocyte subsets in dairy cows with PID after calving.

MATERIALS AND METHODS

Cows: Twenty Holstein cows between 2 and 6 years of age, reared in one herd, were used in this study. We divided the cows into two groups: Group 1 (age; 4.56 ± 0.40 years) consisted of nine cows that experienced infectious diseases (mastitis; n=4, puerperal fever; n=5) within 2 weeks after calving, and Group 2 (age 3.58 ± 0.33 years) consisted of eleven cows that were healthy during the periparturient term. All cows had an easy calving.

Sample collection and analysis: Blood samples were collected from the caudal vein into tubes containing dipotassium-EDTA on the day of calving, at 1 week, and at 1, 3 and 6 months after calving. Total white blood cell counts (WBC) were determined with a blood cell counter (Sysmex-Counter F-800). The absolute leukocyte count was calculated by multiplying the WBC by the observed percentage of lymphocytes or monocytes from the differential count. Concentrations of plasma protein were measured by electrophoresis.

Flow cytometry: Two m*l* of blood samples were mixed with 4 m*l* of 0.83% ammonium chloride solution, and the leukocytes were separated. Leukocyte samples were washed twice in phosphate-buffered saline (PBS). About 2×10^6 cells/m*l* were suspended in PBS, and samples were incubated at 4°C for 60 min in PBS containing monoclonal antibodies to the bovine cell surface marker. The primary antibodies used and a description of the working solutions are given in Table 1. After 60 min incubation the cells were washed twice in PBS, and incubated in PBS with the secondary antibody, which was FITC-conjugated Goat-anti-mouse IgG-fluorescein isothiocyanate (Cappel, Durham, NC, U.S.A.) for 30 min. After incubation, the samples were washed twice with PBS, and then cell surface markers were visualized with goat anti-mouse IgG. The cells were analyzed with a flow cytometer (EPICS ELITE ESP Coulter Corp., Florida, U.S.A.). Data were acquired from 5000 events per sample. In all analyses, mononuclear cells were gated out from other leukocytes subsets based on the dot-plots of their forward scatter (FSC) and side scatter (SSC), as reported previously [13].

Statistical analysis: Differences between the two groups were determined by means of Student's *t*-test. And the data in each group during the observed term were evaluated by One-Way ANOVA for evaluation of the month relative to

Table 1. Antibodies used in the immunostaining of peripheral blood mononuclear leukocytes

Antibodies	Clone	Isotype	Specificity	Source[a,b,c)]
CD3	MM1A	IgG1	Pan T cell	VMRD
CD4	CACT138A	IgG1	Helper/inducer	VMRD
CD8	CACT80C	IgG1	Cytotoxic/suppressor	VMRD
CD14	MY4	IgG1	Monocyte	Coulter
WC1	IL-A29	IgG1	gd T cell	VMRD
IgM		IgG1	B cell	KPL

a) VMRD=VMRD, Inc. (pullman, WA, U.S.A.).
b) Coulter=Coulter Immunology Hailed (Florida, U.S.A.).
c) KPL=Kirkegaard & Perry, Laboratories, Inc. (Gaitherburg, MD, U.S.A.).

periparturient. Values of $P<0.05$ were regarded as significant.

RESULTS

The numbers of WBC and neutrophils were highest on the day of calving in both groups, and were lowest at the end of week one. The numbers of WBC, neutrophils and mononuclear cells were lower in Group 1 than in Group 2 during the period from the day of calving to the end of month 1. Total plasma protein levels peaked at month 1 in both groups, and gradually decreased thereafter. Total plasma protein was significantly higher in Group 1 from month 1 to month 3 than in Group 2 (Fig. 1).

The percentages of $CD3^+$ and $CD4^+$ cells in both groups were lowest on the day of calving, and were increased at the end of month 1. These percentages of $CD3^+$ and $CD4^+$ cells tended to be higher in Group 1 than in Group 2 at the end of month 1. The percentages of $CD8^+$ and $WC1^+$ cells in both groups were lowest on the day of calving, and increased gradually through month 6. The $CD14^+$ percentages in both groups peaked on the day of calving, and then decreased gradually. The percentages of IgM^+ cells in both groups were lowest on the day of calving, and increased gradually through month 6. The percentages of IgM^+ cells in Group 1 tended to be lower during months 1 to 6 than those in Group 2, and significant difference was detected at month 3 (Table 2).

The numbers of $CD3^+$, $CD4^+$ and $CD8^+$ cells in Group 1 tended to be lowest on the day of calving, and then increased gradually up to the end of month 1. There was a significantly higher number of $CD4^+$ cells in Group 1 than in Group 2 at month 1. The numbers of $CD8^+$ cells were significantly lower in Group 1 than in Group 2 at week 1. In Group 2, these numbers were stable during the observed term. The $CD4^+/CD8^+$ ratio in Group 1 increased from the day of calving up to the end of month 1, and then decreased through month 6. There was a significant difference in this ratio between the two groups at month 1. $WC1^+$ cells were lowest on the day of calving and then increased continuously in both groups up to month 6 (Fig. 2). The numbers of $CD14^+$ cells in Group 1 peaked on the day of calving, and then decreased to week the end of 1, and these numbers

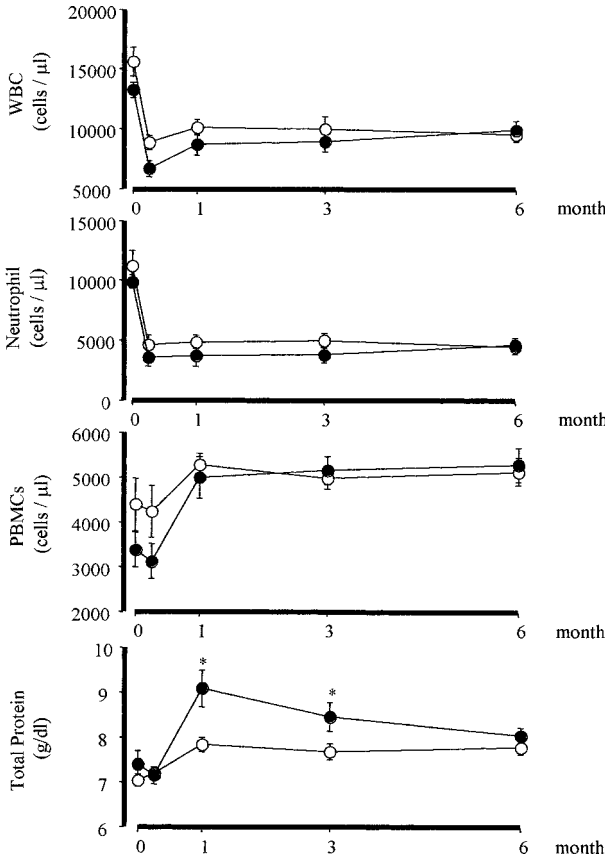

Fig. 1. Changes in leukocytes and total protein after calving in two groups. Month 0 means calving day. Gro u ● 1 (), Gr (○), Mean ± S.E., *; $P<0.05$.

remained stable from month 1 to month 6. A significant decrease in these numbers in Group 1 contrasted with the healthy group at the end of week 1. In both groups, the numbers of IgM^+ cells were lowest on the day of calving and then gradually increased. These numbers in Group 1 remained lower than in Group 2 during the observed term, and there was a significant difference at month 3 (Fig. 3).

Significant changes were detected in the WBC, CD3,

Table 2. Percentage of each leukocyte after calving

	month	Group 1 (n=9)	Group 2 (n=11)	
$CD3^+$	0	25.80 ± 2.91	30.14 ± 3.67	
	0.2	30.42 ± 4.85	33.58 ± 3.20	
	1	44.81 ± 3.02	37.66 ± 2.62	
	3	34.58 ± 3.63	35.70 ± 3.85	
	6	44.17 ± 1.84	41.13 ± 1.88	
$CD4^+$	0	13.73 ± 1.33	15.31 ± 1.90	
	0.2	16.57 ± 2.64	17.27 ± 1.68	
	1	25.14 ± 2.72	18.84 ± 1.77	
	3	16.89 ± 2.51	19.72 ± 2.31	
	6	19.76 ± 1.80	17.21 ± 0.84	
$CD8^+$	0	7.33 ± 1.23	8.65 ± 1.14	
	0.2	6.84 ± 0.79	9.51 ± 1.20	
	1	11.13 ± 1.66	11.14 ± 0.63	
	3	10.13 ± 1.09	13.02 ± 1.69	
	6	14.32 ± 1.52	15.47 ± 1.28	
$WC1^+$	0	5.38 ± 1.32	6.51 ± 1.19	
	0.2	7.61 ± 2.12	7.10 ± 1.77	
	1	5.77 ± 1.84	7.29 ± 1.31	
	3	6.64 ± 0.85	7.52 ± 0.93	
	6	8.81 ± 1.77	10.03 ± 1.50	
$CD14^+$	0	38.09 ± 2.58	34.09 ± 4.29	
	0.2	22.23 ± 3.00	24.38 ± 2.33	
	1	21.60 ± 3.27	22.64 ± 2.52	
	3	26.61 ± 3.48	23.97 ± 2.92	
	6	19.02 ± 2.24	15.36 ± 2.22	
IgM^+	0	19.94 ± 2.21	18.68 ± 2.21	
	0.2	24.38 ± 2.07	24.45 ± 2.14	
	1	24.72 ± 3.40	28.96 ± 2.55	
	3	24.98 ± 2.09	36.21 ± 5.18	*
	6	34.46 ± 3.74	45.50 ± 5.03	

Values (%) are the mean ± SE.
* means the significant different between two groups ($p<0.05$).

CD4, CD8, CD4/CD8 rate, Ht and TP in Group 1 during the observed term. In Group 2, there were significant changes in WBC and neutrophils, but the changes in the other values were stable and not significant.

DISCUSSION

Most of the information available regarding changes in T cells in cows with PID around the time of calving have been derived from investigations in healthy cows. Our present study found that the numbers of T cells, such as $CD3^+$, $CD4^+$ and $CD8^+$ cells, tended to be lower in the cows with PID than in healthy cows during the first week after calving. T cells can produce cytokines, and several cytokines induce the division and differentiation of T cells. During the periparturient period in cows, the production of IFN-gamma in bovine peripheral blood mononuclear cells (PBMCs) was depressed [4]. The $CD4^+$ lymphocytes act predominantly as T-helper-2 cells as opposed to T-helper-1 cells during the first three days after calving [16]. In other words, physiological changes involving diminished cellular immune response occur in cows during calving. The diminished T cells in PID cows might lead to a severe decline in T-cell function on the day of calving, prior to the occurrence of PID. In particular, the number of $CD8^+$ T cell decreased at week 1 in the PID cows. This observation was similar to that of a previous result reporting lower peripheral $CD8^+$ T cells in the first 50 days after delivery [21]. $CD8^+$ T cells have been demonstrated to increase over time in postpartal women, and there is also an increase in cytotoxic activity in early postpartal term [19]. This finding indicated that the cytotoxic activity of $CD8^+$ T cells in the PID group might be further depressed during the early postpartal period. Although the numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T cells tended to be low in the PID group, the changes in $WC1^+$ T cells followed a similar pattern in both groups after calving. This finding may indicate that gamma delta T cells did not play a pivotal role in host defense or onset of PID.

After delivery, helper T cells and cytotoxic T cells were reported to increase from 1 to 4 months postpartum in

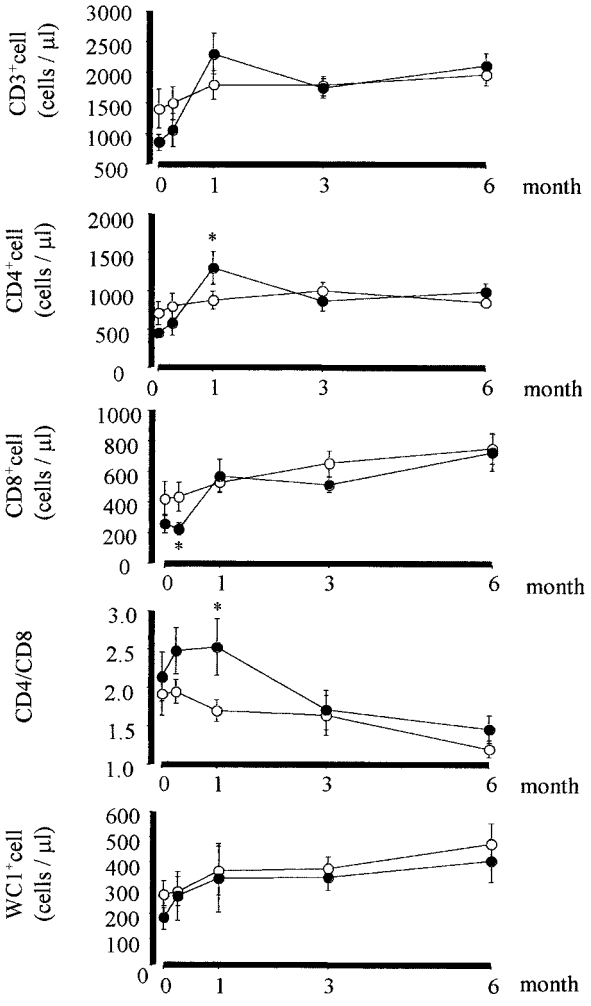

Fig. 2. Changes in leukocytes after calving in two groups. Month 0 means calving day. Group 1 (●), Group 2 (○), Mean ± S.E., *; P<0.05.

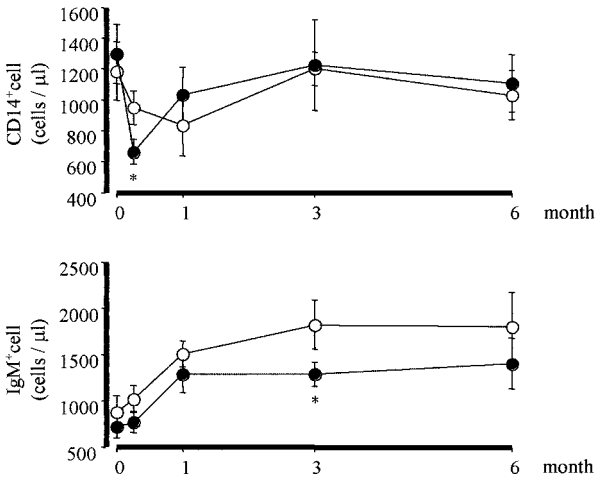

Fig. 3. Changes in leukocytes after calving in two groups. Month 0 means calving day. Group 1 (●), Group 2 (○), Mean ± S.E., *; P<0.05.

humans [22]. This finding has been suggested to indicate an increase in the postpartum woman's ability to mount a defense against pathogens. Our study found a significant increase in the $CD4^+/CD8^+$ ratio after an increase in the number of $CD4^+$ T cell in the PID group at the end of month 1. The reported increase in the numbers of alpha beta T cells observed in milk from cows with staphylococcal mastitis was primarily due to increased numbers of $CD4^+$ T cells [18], and increased IL-2 and IL-4 production were correlated with the high $CD4^+$ T lymphocyte proportions [9]. The increased $CD4^+/CD8^+$ ratio at the end of month 1 in cows with PID might appear to be a result of activated T cell function, involving cytokines after some type of infection.

A peculiar change in $CD14^+$ monocytes was observed in the PID group. A decreased percentage of $CD14^+$ monocytes were observed in patients with infectious shock [17]. The $CD14^+$ macrophages/monocytes ratio is considered due to the cells secreting inflammatory cytokines, suggesting that the release of such factors induces an inflammatory response. The occurrence of metritis was reported to be closely related to retained placenta [1], and further, the phagocytotic activity of macrophages was decreased conspicuously within 1 day after calving in cows with retained placenta [10]. It was possible to relate the temporary decrease in $CD14^+$ monocytes to the inflammatory response in the PID group, but the mechanism of $CD14^+$ monocyte response at week 1 was unclear.

The lowered number of peripheral IgM^+ cells during months 1 to 6 in PID cows followed significantly increased levels of plasma protein. Increased serum protein was accompanied by an enhanced total protein concentration after increases in γ-globulin in cows with chronic mastitis [14]. A decrease in B cells was reported previously in cows with mastitis [24]. These findings suggested that there was a chronic inflammatory response in Group 1 during months 1 to 6. The decrease in B cells in the peripheral blood has been suggested as one of the characteristic immune responses in chronic inflammatory diseases.

During the postcalving period, there was a marked reduction in immune activation caused by serious stress [11]. Increased peripheral leukocytes and decreased PBMCs seen after calving are attributable to physiological stress, which induces leukocytosis in cows within the first postpartal week [2]. The cows with mastitis showed prolonged increases in plasma cortisol, indicating increased stress hormone during the 2 days after calving [12, 20]. Corticosteroids can inhibit translocation of transcription factor, and induce variable immunosuppression. The immunosuppressive effects of cortisol were suspected to be one of the causative agents of immunosuppression during the periparturient term in cows with PID.

We reported changes in the leukocyte subset in the cows

with PID after calving, but the difference in immune response between mastitis and puerperal fever was not clarified. Kimura [7] showed a noticeably lower neutrophil function before parturition in cows with retained placenta. Previous investigations suggested that pregnancy-induced immunosuppression is a natural mechanism during pregnancy [5]. T lymphocyte subsets change dynamically during the various stages of lactation [15, 23]. These investigations seemed to suggest the possibility that the cows with PID had serious disorders of the cellular immune system before calving. Recently, the effects of the onset of lactation on changes in PBMCs populations in mastectomized cows were reported [6]. In that investigation, no drastic changes in peripheral T cells and monocytes were detected in mastectomized cows during the calving period. It appeared that change in the immune status of the mammary gland was one of the factors affecting the onset of PID during the periparturient period. In conclusion, we suspect that a decrease in T cells at calving may be linked to systemic immunosuppression, and leads to the appearance of infectious diseases in dairy cows.

REFERENCES

1. Bruun, J., Ersboll, A.K. and Alban, L. 2002. Risk factors for metritis in Danish dairy cows. *Prev. Vet. Med.* **54**: 179–190.
2. Convey, E.M. 1974. Serum hormone concentrations in ruminants during mammary growth, lactogenesis, and lactation: a review. *J. Dairy Sci.* **57**: 905–917.
3. Hagiwara, E., Abbasi, F., Mor, G., Ishigatsubo, Y. and Klinman, D.M. 1995. Phenotype and frequency of cells secreting IL-2, IL-4, IL-6, IL-10, IFN and TNF-alpha in human peripheral blood. *Cytokine* **7**: 815–822.
4. Ishikawa, H., Shirahata, T. and Hasegawa, K. 1994. Interferon-gamma production of mitogen stimulated peripheral lymphocytes in perinatal cows. *J. Vet. Med. Sci.* **56**: 735–738.
5. Iwatani, Y., Amino, N., Tachi, J., Kimura, M., Ura, I., Mori, M., Miyai, K., Nasu, M. and Tanizawa, O. 1988. Changes of lymphocyte subsets in normal pregnant and postpartum women: postpartum increase in NK/K (Leu 7) cells. *Am. J. Reprod. Immunol. Microbiol.* **18**: 52–55.
6. Kimura, K., Goff, J.P., Kehrli, M.E. Jr., Harp, J.A. and Nonnecke, B.J. 2002. Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. *J. Dairy Sci.* **85**: 1437–1444.
7. Kimura, K., Goff, J.P., Kehrli, M.E. Jr. and Reinhardt, T.A. 2002. Decreased neutrophil function as a cause of retained placenta in dairy cattle. *J. Dairy Sci.* **85**: 544–550.
8. Kimura, K., Goff, J.P., Kehrli, M.E. Jr. and Harp, J.A. 1999. Phenotype analysis of peripheral blood mononuclear cells in periparturient dairy cows. *J. Dairy Sci.* **82**: 315–319.
9. Mitchell, L.C., Davis, L.S. and Lipsky, P.E. 1989. Promotion of human T lymphocyte proliferation by IL-4. *J. Immunol.* **142**: 1548–1557.
10. Miyoshi, M., Sawamukai, Y. and Iwanaga, T. 2002. Reduced phagocytotic activity of macrophages in the bovine retained placenta. *Reprod. Domest. Anim.* **37**: 53–56.
11. Nanda, A.S., Dobson, H. and Ward, W.R. 1990. Relationship between an increase in plasma cortisol during transport-induced stress and failure of oestradiol to induce a luteinising hormone surge in dairy cows. *Res. Vet. Sci.* **49**: 25–28.
12. Nikolic, J.A., Kulcsar, M., Katai, L., Nedic, O., Janosi, S. and Huszenicza, G. 2003. Periparturient endocrine and metabolic changes in healthy cows and in cows affected by mastitis. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.* **50**: 22–29.
13. Ohtsuka, H., Komatsu, S., Konnai, S., Fukuda, S., Kiku, Y., Yoshino, T., Koiwa, M. and Kawamura, S. 2002. Comparison of peripheral leukocytes in Japanese Black and Holstein calves. *J. Jpn. Vet. Med. Assoc.* **55**: 789–795.
14. Ohtsuka, H., Kudo, K., Mori, K., Nagai, F., Hatsugaya, A., Tajima, M., Tamura, K., Hoshi, F., Koiwa, M. and Kawamura, S. 2001. Acute phase response in naturally occurring coliform mastitis. *J. Vet. Med. Sci.* **63**: 675–678.
15. Ohtsuka, H., Koiwa, M., Sato, Y., Yamashita, M., Takada, T., Itoh, N., Hoshi, F., Yoshino, T. and Kawamura, S. 2001. Relationship between serum total cholesterol and post-calving peripheral leukocytes in healthy Dairy cows. *J. Jpn. Vet. Med. Assoc.* **54**: 761–765.
16. Shafer-Weaver, K.A., Corl, C.M. and Sordillo, L.M. 1999. Shifts in bovine CD4+ subpopulations increase T-helper-2 compared with T-helper-1 effector cells during the postpartum period. *J. Dairy Sci.* **82**: 1696–1706.
17. Skrzeczynska, J., Kobylarz, K., Hartwich, Z., Zembala, M., Pryjma, J. 2002. CD14+CD16+ monocytes in the course of sepsis in neonates and small children: monitoring and functional studies. *Scand. J . Immunol.* **55**: 629–638.
18. Soltys, J. and Quinn, M.T. 1999. Selective recruitment of T-cell subsets to the udder during staphylococcal and streptococcal mastitis: analysis of lymphocyte subsets and adhesion molecule expression. *Inf. Immun.* **67**: 6293–6302.
19. Stagnaro-Green, A., Roman, S.H., Cobin, R.H., el-Harazy, E., Wallenstein, S. and Davies, T.F. 1992. A prospective study of lymphocyte-initiated immunosuppression in normal pregnancy: evidence of a T-cell etiology for postpartum thyroid dysfunction. *J. Clin. Endocrinol. Metab.* **74**: 645–653.
20. Van Kampen, C. and Mallard, B.A. 1997. Effects of peripartum stress and health on circulating bovine lymphocyte subsets. *Vet. Immunol. Immunopathol.* **59**: 79–91.
21. Waage, S., Odegaard, S.A., Lund, A., Brattgjerd, S. and Rothe, T. 2001. Case-control study of risk factors for clinical mastitis in postpartum dairy heifers. *J. Dairy Sci.* **84**: 392–399.
22. Watanabe, M., Iwatani, Y., Hidaka, Y., Mitsuda, N. and Amino, N. 1996. Changes in soluble CD4 and CD8 proteins in healthy pregnant and postpartum women. *Am. J. Reprod. Immunol.* **36**: 220–227.
23. Yang, T.J., Ayoub, I.A. and Rewinski, M.J. 1997. Lactation stage-dependent changes of lymphocyte subpopulations in mammary secretions: inversion of CD4+/CD8+ T cell ratios at parturition. *Am. J. Reprod. Immunol.* **37**: 378–383.
24. Yang, T.J., Mather, J.F. and Rabinovsky, E.D. 1988. Changes in subpopulations of lymphocytes in peripheral blood, and supramammary and prescapular lymph nodes of cows with mastitis and normal cows. *Vet. Immunol. Immunopathol.* **18**: 279–285.