Relationship Between Parity and Cellular Composition of Somatic Cells in Milk of Chinese Holstein Cows

A.B. Yu, G.Q. Zhao, S.Q. Tian and Y.J. Huo
Laboratory of Animal Nutrition, College of Animal Science and Technology,
Yangzhou University, Yangzhou, P.R. China

Abstract: The present study was made to explore the relationship between the cellular composition of somatic cells in milk of dairy cattles and parity. A total of 75 Chinese Holstein cows (from primiparous to 5th lactation) from Experimental Farm of Yangzhou University were selected and divided into 5 groups according to parity (15 cows per group). Composition of leukocytes in blood and concentrations of fat, protein, lactose, total solid, Milk Urea Nitrogen (MUN) and Somatic Cells Counts (SCC) in the milk were determined. The ratios of Macrophages (MAC), Polymorphonuclear Neutrophil Leucocytes (PMN) and Lymphocytes (LYM) in milk and peripheral blood were examined using flow cytometry and whether the ratios of the three kinds of cells was influenced by parity of the cow was then analyzed. Results showed that the percentages of MAC, PMN and LYM were 5.72~7.26, 30.10~41.10 and 50.65%~60.12% in blood, respectively and showed no significant difference among groups. The somatic cells in milk were mainly composed of MAC, PMN and LYM. The range of MAC, PMN and LYM were 11.47~22.56, 19.48~29.56 and 45.83%~63.44%. With the increase of parity, the ratios of MAC and PMN tended to be increased and LYM tended to be decreased. The results suggested that the progressive increase of SCC when parity increased might be likely related to immune mechanisms in absence of infection and resulted from increased secretion of MAC and PMN in udder. The changes of the ratios of different leukocytes to SCC in milk might provide a sensible method of indicating mammary health condition without visible infections and further understanding of generation of SCC in milk in response to parity.

Key words: Dary cattle, parity, somatic cells counts, leukocyte, milk, China

INTRODUCTION

The presence of cells in bovine milk, so called somatic cells has been recognized and studied for many years. Somatic Cell Count (SCC) is the total number of leukocytes cells per milliliter in milk (Miller *et al.*, 1986). SCC in the milk of a healthy cow is normally $2~3 \times 10^5$ mL^{-1} and is often used as an indirect measure of mammary infection status (Shook and Schultz, 1994; Caraviello *et al.*, 2005). These cells originate from blood cells and play an important role in the metabolism of the mammary gland (Burvenich *et al.*, 1994). The majority of the cells in somatic cell counts are leukocytes and others are cells from the udder secretory tissue (epithelial cells) (Bradley and Green, 2005). The epithelial cells are shed and renewed in the normal body processes. The white blood cells serve as a defense mechanism to fight disease infection and assist in repairing damaged tissue (Ma *et al.*, 2000). The white blood cells are mainly composed of Macrophage (MAC), Lymphocyte (LYM) and Polymorphonuclear Neutrophil leukocytes (PMN) (Dosogne *et al.*, 2003; Bradley and Green, 2005).

Morgante *et al.* (1996) reported the SCC from healthy primiparous Comisana ewes was $56.7 \pm 45.2 \times 10^3$ mL^{-1} consisting of 30.6% PMN, 57.3% MAC and 8.2% LYM. However, reports about the cellular composition of somatic cells in bovine milk are rare and there is no evidence to indicate the same composition that occurs in ewe's milk occurs in the dairy cow. Paape and Capuco (1997) claimed that neutrophils made up 50-70% of the somatic cell count in milk from goats free of intramammary infection whereas neutrophils only made up 5-20% of the total cell count in bovine milk. Milk leukocytes play an important role in defending the mammary gland from aggression of pathogenic microorganisms. The number and relative proportion of each type of leukocyte is influenced by various physiological and pathological states of the udder (Cuccuru *et al.*, 1997).

A lot of factors such as season, parity, lactation stage, nutrition, sanitation, environment, management and genetic factors could influence the level of SCC in bovine milk (Green *et al.*, 2006, 2008; Heuven *et al.*, 1988; Sheldrake *et al.*, 1983). High levels of SCC in milk could result in great loss of milk yield and have negative effect

on quality, life time and processing traits of milk (Barbano *et al.*, 1991; Jones *et al.*, 1984). SCC is mainly composed of leukocytes produced by the cow's immune system to fight an inflammation in the mammary gland or mastitis. SCC provides an indication of the healthy condition of mammary gland in an individual cow or in the herd if bulk milk is used and it is a hot topic in recent studies.

Previous studies mostly concentrated on the relationship between SCC and the factors that affect their occurrence (Ceron-Munoz *et al.*, 2002; Durr *et al.*, 2008; Rekik *et al.*, 2008; Schepers *et al.*, 1997). Few studies have reported about the cellular composition of SCC secreted from dairy cow mammary. The purpose of the study was to characterize the cellular composition of SCC in milk in related to different parity in dairy cows.

MATERIALS AND METHODS

Experimental animals: Seventy five healthy Chinese Holstein cows with five different parity (1st-5th lactation) from Experimental Farm of Yangzhou University were selected in the study. Each parity group had 15 cows. Body weight, lactation stage and milking yield of the cow at the beginning of the trial were considered before being selected. Each cow was examined to check for the presence of redness, swelling, hardness or pain in the udder or the presence of clots in the milk to ensure all the animals selected had shown without mastitis. All the cows were housed in the same type of free stall barns and fed the same Total Mixed Ration (TMR) 3 times daily. The cows were milked 3 times daily. Body weight, milking yield and lactation stage were 588.37±31.21 kg, 22.31±3.13 kg day^{-1} and 29.17±5.45 days, respectively.

Milk composition analysis: Duplicate milk samples from each cow (4:3:3 mixture of morning, noon and evening milk by volume) were collected on the sampling day. Samples were collected on 3 continuous days with 3 samples taken per cow. Therefore, total 45 samples of each group were collected for SCC composition analysis and each milk samples (50 mL) was preserved with potassium dichromate and analyzed for fat, protein, lactose, total solids, Milk Urea Nitrogen (MUN) and Somatic Cell Count (SCC) by using a MilkoScan Minor machine (MilkoScan 4000, Foss Electric, Hillerod, Denmark).

Blood sample and milk sample's collection for flow cytometry analysis: Blood samples were taken before the morning milking at 8.00 am from the coccygeal vein and

heparin sodium was used as anticoagulant in the sample tubes. Each sample was 10 mL in volume and was kept in an ice box prior to delivery to the lab. The 2 mL of each blood sample was taken and prepared for the analyses of leukocyte composition. The differential leukocyte count milk samples were foremilk samples collected before the morning milking. Each milk sample was 1000 mL in volume. Blood and milk samples were collected on different sampling day and collected at a time so total 75 samples of blood or milk were collected for cytmetry analysis.

Blood sample and milk sample's preparation for flow cytometry analysis: Preparation of each blood sample was as follows: Isolation of somatic cells (including white and red cells) and cell counting: 2 mL blood sample was layered on 20 mL of PBS in a 50 ML centrifuge tube and centrifuged at 2300×g for 15 min at 4°C and the supernatant was subsequently discarded. This operation was repeated 2-3 times to isolate and recover the total cell pellets of blood sample. After dilution with 1 mL PBS, the cells were percolated with a 400 mesh strainer to perform a microscopic slide cell count (Olympus CKX41); rinse with buffer and labeling: Suspended the cells with 1 mL PBS and 1 mL 10% bovine serum (Fetal bovine serum, FBS) then centrifuged at 2300×g for 15 min at 4°C and the supernatant was subsequently discarded. Blotted 400 μL cell deposition into 1.5 mL centrifuge tube, suspended with 1 mL PBS and 1 mL 10% bovine serum and after that the cells were labeled with 10 μL CD14 (FITC MCA2678F AbD Serotec) and 10 μL CD45 (RPE MA1-81458 Thermo Scientific) at the same time. They were incubated for 40 min and then centrifuged at 2300×g for 15 min at 4°C; deterioration of red cells and rinse of white cells: 1 mL erythrocyte lysate was added to the cell pellets and centrifuged at 2300×g for 15 min at 4°C after 20 min incubation. The cell deposition was rinsed 3~4 times with 1 mL PBS for further flow cytometric sorting (BD FACSCalibur).

Preparation of each milk sample was similar to the preparation of blood sample. But before that of it, each milk sample (200 mL) collected from each cow was centrifuged at 4500×g for 20 min at 4°C to remove the fat.

Flow cytometry and cell sorting: Flow cytometry identification of cells based on forward and side scatter is difficult because cellular debris may interfere with the scatter pattern of normal cells. This is especially true for bovine milk involved with impurity substances and phagocytosis of milk components may alter both size and intracellular granularity. Therefore, fluorescent labeling techniques with monoclonal antibodies CD45 (RPe) and

CD14 (FITC) have been developed based on a previous study (Dosogne *et al.*, 2003) to differentiate leukocytes in both blood and milk.

A flow cytometer (BD FACSCalibur) equipped with an argon ion laser was used for determining the differential leukocyte count. The excitation wavelength of the laser was 495 and 565 nm. The forward scatter signal was amplified before side scatter and fluorescence signals were not amplified. The signal was measured with a band-pass filter and registered on a log scale. The leukocyte could be classified as LYM, PMN and MAC based on the size, inner complex structure of the cell pellets and intensity of fluorescence of different wavelengths.

Statistical analysis: Results were expressed as means±standard errors of the means. The data were analyzed by one-way ANOVA and a Duncan's test was used to determine the statistical significance of differences between parity groups of cows. High and mostly high significance levels were assumed at $p<0.05$ and $p<0.01$, respectively.

RESULTS AND DISCUSSION

Milk composition: A total of 225 samples were analyzed for milk composition. The results are shown in Table 1. It was shown that the parity did not influence the percent fat, protein, total solid and MUN in milk during the early lactation significantly. But the percent of lactose of primiparous was significantly higher than that of multiparous ($p<0.05$). When the relationship between milk SCC and parity was compared, data showed that SCC was markedly increased as parity rose. Meanwhile, milk SCC of primiparous was significantly lower than that of 4th and 5th parity ($p<0.05$).

Categorization of leukocytes in blood and milk: Leukocytes cells were isolated from peripheral blood and milk for the flow cytometric cell sorting procedure. To better characterize the leukocyte cells, researchers experimented to find the appropriate antibody and labeling condition or circumstance through the trial. Flow cytometric differential leukocyte count of samples after

antibody labeling was quantified using fluorescence dot plot. Three different cell types were separated obviously in different regions in both blood and milk sample analysis results. MAC (P2), PMN (P3) and LYM (P4) were defined as main cell types in blood and LYM (P3), MAC (P4), PMN (P5) in milk. In addition, epithelial cell in both blood and milk could not be separated through this method. Flow cytometric analysis results of blood and milk are shown in Fig. 1 and 2, respectively.

Composition of leukocytes in blood: The proportion of MAC in peripheral blood obtained from different parity of cows ranged from 5.72-7.26%. The proportion of PMN ranged from 30.10-41.10% and LYM varied from 50.65-60.12%. The percent of MAC, LYM and PMN was relatively stable and showed no significant difference within each of the parities ($p>0.05$).

Composition of leukocytes in milk: Analysis results of the leukocyte composition in milk showed differences in variation compared to that of blood. The proportion of MAC in milk ranged from 11.47-22.56% and showed increasing trend in line with the parity. Percent of MAC on SCC in 1st parity was mostly low than that of 4th and 5th parity ($p<0.01$). The ratio of MAC in 2nd was significantly <4 and 5th parity at the same time ($p<0.05$). The proportion of PMN ranged from 19.48-29.56% and showed an increasing trend along with the parity and reached maximum on 5th parity. It was showed that percent of PMN in primiparous was low significantly than 4th parity ($p<0.05$) as well as mostly low than that of 5th parity ($p<0.01$). The proportion of LYM varied from 45.83-63.44%. The percent of LYM tended to decrease with increasing parity and reached a minimum value on the 4th parity. Value of LYM in primiparous cattle was mostly high than 5th parity ($p<0.01$) and showed a significant difference to 4th parity ($p<0.05$). In addition, values between 2nd and 5th parity also showed significant difference ($p<0.05$).

The result of this study suggested that parity of cows almost did not affect the traits of milk but SCC and elevation of parity will be result in an increase of SCC. This result was agreed with Wilson *et al.* (1995) and Dulin *et al.* (1983) which reported that with increasing parity the somatic cell count in milk also increased in dairy cows. Zeng and Escobar (1995) also claimed that parity of goats did not affect variables of composition of milk including percent fat, percent protein, percent lactose, percent solids non-fat and SCC except for milk production. However, Laevens *et al.* (1997) reported that significant effects of parity on SCC in milk could not be found when only bacteriologically negative cows were considered. Thus, the effect of parity on variation of SCC was likely due to incidental factors such as mastitis infections, just

Table 1: Composition of milk from cows in relation to parity

Items	Parity[1]					SEM[2]
	1st	2nd	3rd	4th	5th	
Fat (%)	3.68	3.75	3.80	3.92	3.92	0.05
Protein (%)	3.30	3.32	3.33	3.25	3.42	0.03
Lactose (%)	5.04[a]	4.76[b]	4.74[b]	4.72[b]	4.70[b]	0.02
Total solid (%)	13.12	13.10	13.09	13.21	13.20	0.07
MUN (mg dL^{-1})	15.01	14.44	14.30	14.57	14.87	0.34
SCC×10^3	265.29[a]	367.90[ab]	374.58[ab]	449.50[b]	456.26[b]	22.76

[a, b]Means within a row with different superscripts differ ($p<0.05$). [1]Parity: Samples were collected from 5 different parity herds on 3 continuous days with 3 samples taken per cow. Total 225 (= 15×5×3) samples included for analysis. [2]SEM = Standard Error of the Mean
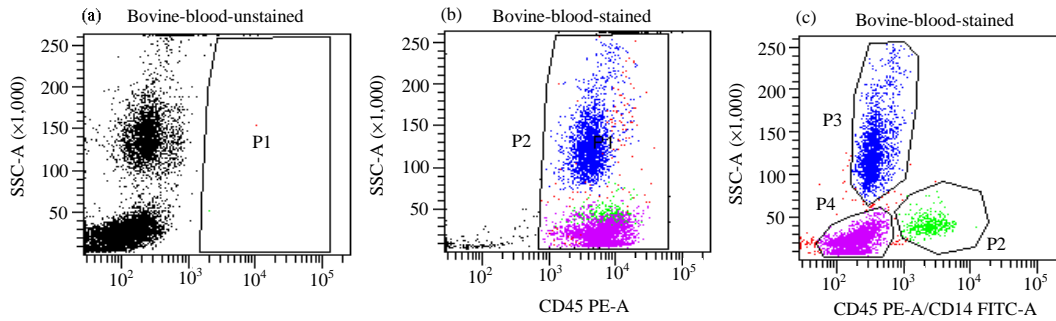
Fig. 1: Flow cytometric analysis of fluorescence antibody labeled bovine blood cells. (a) All leukocytes were unlabeled and all of them located outside the gate P1. (b) All leukocytes were labeled with CD45. This resulted in the increased intensity of fluorescence of cells with the cells mainly in gate P1 area and only few impurity substances or segment of cells remained outside of the gate and were in the district P2. Blue fluorescence showed PMN has been isolated from leukocytes based on Side Scatter (SSC) of flow cytometric cell sorting. (c) All leukocytes were labeled with CD45 and CD14 and MAC was labeled with CD14 specifically. Subsequently, leukocytes were distributed in three areas P2 (MAC), P3 (PMN) and P4 (LYM), distinctively
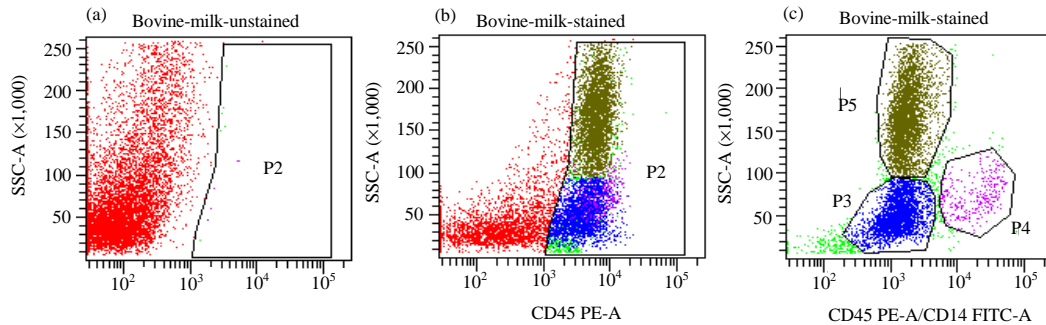

Fig. 2: Flow cytometric analysis of fluorescence antibody labeled bovine milk somatic cells. (a) All leukocytes were unlabeled and all of them located outside the gate P2. (b) All leukocytes were labeled with CD45. This resulted in the increased intensity of fluorescence of cells and with the cells mainly in gate P2 area. Yellow fluorescence showed PMN has been isolated from leukocytes based on Side Scatter (SSC) of flow cytometric cell sorting. (c) All leukocytes were labeled with CD45 and CD14 and MAC was labeled with CD14 specifically. Subsequently, leukocytes were distributed in three areas P3 (LYM), P4 (MAC) and P5 (PMN), distinctively

as Heuven *et al.* (1988) claimed the increase of SCC over parities shows that older cows were more likely to suffer prolonged elevation of SCC or even permanent udder damage. High SCC of older cows in milk could also be explained by physiological reduction of milk yield (Cuccuru *et al.*, 1997). Therefore, in order to learn more about the reason of rising of SCC over parities, it was necessary to classify the composition of somatic cells in milk.

It is generally considered that somatic cells of milk originate from the blood and the number, differential count and function of leukocytes within the mammary gland could contribute to the defense against invading pathogens (Mullarky *et al.*, 2001; Zecconi *et al.*, 1994). PMN are produced in the bone marrow and migrate into body tissues constituting a physiologic barrier to microbial invasion. There, they phagocytize, kill and digest bacteria in a complex but sophisticated manner utilizing energy and granular constituents (Morgante *et al.*, 1996). During this process PMN also release certain agents which by themselves are inflammatory (Jones *et al.*, 1984). As physiological and environmental factors have a smaller influence on the number of PMN in milk (Jones *et al.*, 1984) since the increase is related strictly to the presence of microorganisms in the mammary gland (Cuccuru *et al.*, 1997).

Therefore, the increase percent of PMN in milk observed in this study confirms the woresen of mammary inflammation in older cows without visiable mastitis. Considering the count of somatic cells in milk was increased with parity, the amount of PMN secreted cells in the mammary gland of older dairy cows was relatively huge. Increased levels of somatic cell counts in older cows could be explained by remarkable increase of PMN to some extent (Fig. 3).
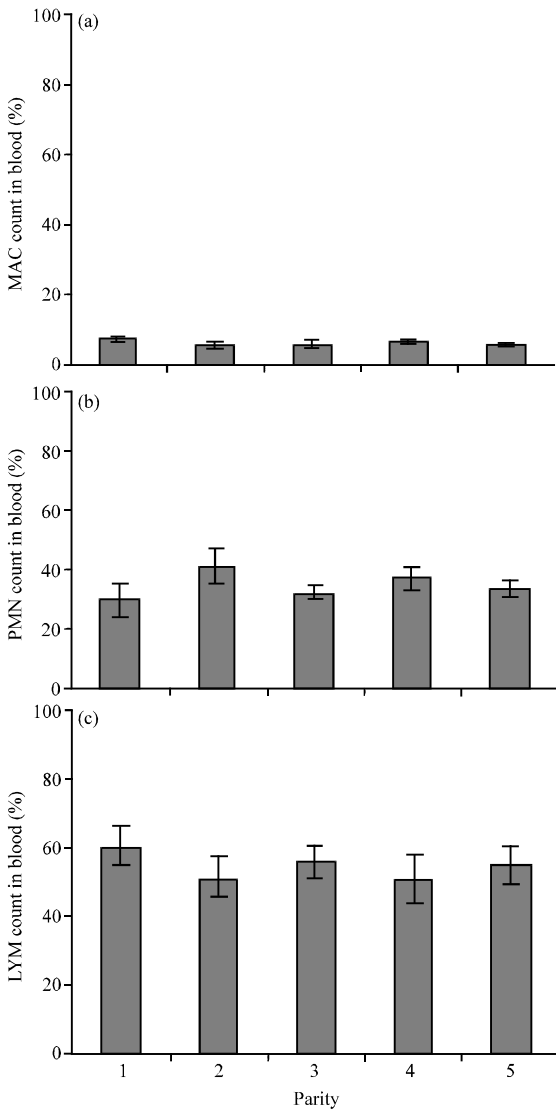
Fig. 3a-c: Differential blood leukocyte percent for cows (n = 75) without clinical sings of mastitis in relation to parity. The bars have no markers means the group have no significant difference (p>0.05) at the 95% confidence interval with two tail t-test
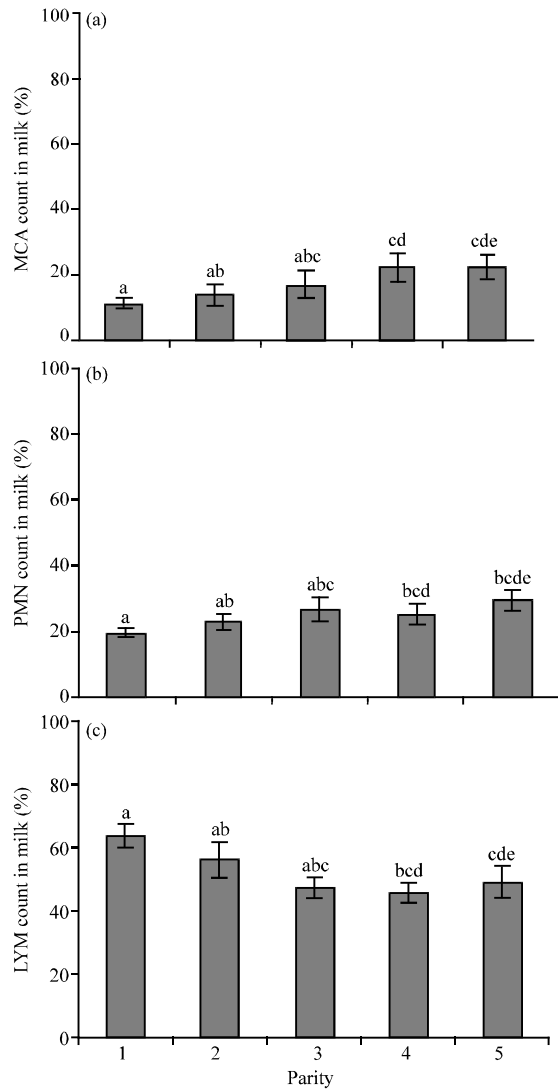
Fig. 4a-c: Differential milk leukocyte percent for cows (n = 75) without mastitis in relation to parity. a-e, the bars with different letters marked means have significant between the groups (p<0.05) and the bars marked have same letters means the group have no significant difference at the 95% confidence interval with two tail t-test

The study found that MAC constituted an important percentage of the somatic cells in milk and was more abundant in milk than that in blood. Furthermore, the level of MAC in milk was increased in multiparous cattle. It could be hypothesized that the higher level of MAC in milk than blood was associated with mobilization of MAC which permeated from blood to the mammary gland through blood capillaries. But the evaluation of dynamics of MAC needs further research. The exact role of MAC in the mammary gland secretions with respect to resistance to mastitis has also not been clearly elucidated.

Denis *et al.* (2006) reported that milk MAC from lactating cows did not exert bactericidal activity against *S. uberis* and released low quantities of TNF-a in response to this pathogen. Conversely, Sordillo *et al.* (1987) claimed that MAC in mammary gland could secret cytokine and leukotriene that were able to induce PMN movement from blood into mammary gland. Both MAC and PMN were increased in bovine milk in relation to parity in the study was in agreement with this point (Fig. 4).

LYM included T cells, B cells and NK cells which have their own killing ability and secreting of antibody to

defend, kill and clear pathogenic bacteria (Mullarky *et al.*, 2001). In fact, the percent of LYM in milk was observed decrease with increasing parity in the study. For migratory population of LYM is being selectively recruited to the mammary gland from the peripheral and infiltration of the mammary gland by LYM is closely related to the immune response (Harp *et al.*, 2004). In addition, LYM vary in cell populations and their release of important cytokines can influence the immune response and the susceptibility to disease (Meglia *el al.*, 2005). This finding indicated an older cow could have poorer udder immunity status as compared to a primiparous one.

The variation of leukocyte cellular composition of blood covered a narrower field compared to that of milk and showed no significant difference between each of the parities. In other words, the composition of MAC, PMN and LYM in blood was relatively stable and influenced by few factors. In fact, it has been observed that the proportion of MAC on SCC in milk was mostly high than that in blood and the proportion of PMN and LYM was low than that in blood on contrary. What's the relationship between in milk and in blood as leukocyte cellular composition concerned has rare been reported. Burvenich *et al.* (1994) reported that with the acceleration of apoptosis of MAC and LYM in blood of bovine, the susceptibility to disease increases at the same time and more PMN is secreted in blood to resist pathogenic bacteria. But the mechanism of this phenomenon has not been clearly explained and need more evaluation.

CONCLUSION

The Macrophage (MAC), Polymorphonuclear Neutrophil (PMN) and Lymphocyte (LYM) in peripheral blood and milk of healthy cows were separated through fluorescence antibody labeling using flow cytomety. The composition of leukocytes in peripheral blood had no relation to parity in statistics but that of milk was markedly influenced by parity. With increasing of parity, the levels of PMN and MAC showed a tendency to be increased and LYM showed a decreasing trend in milk. Because of a limited numbers of cows recruited in the study, additional research is needed.

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