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Leukocyte Markers Differentiate Non-Infected from Spontaneously Infected Dairy Cows

Do-Hyeon Yu, Jong-Hyeon Lee, Ru-Hui Song, Dong-Ho Noh, Ying-Hua Li, Mi-Jin Lee and Jin-Ho Park[1]

College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, Korea

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Abstract : Spontaneously infected and non-infected dairy cows were assessed in a cross-sectional study aimed at determining whether bovine leukocyte markers may diagnose intra-mammary infections (bovine mastitis). Animals located in herds where bovine mastitis was highly prevalent were investigated (n = 31 animals). The expression of three cell-surface markers (CD11b, CD4 and CD8) was assessed, and the somatic cell count (SCC) and bacteriological analyses (both cultures and PCR tests) were also conducted. Cows identified as infected revealed statistically significant higher milk leukocyte CD11b, CD4 percentage and milk CD4/CD8 ratios than non-infected cows. Immunological markers may diagnose spontaneous bovine mastitis.

Key words **:** Bovine mastitis, Flow cytometry, CD11b/CD4/CD8.

Introduction

The time between an infective agent's invasion of a susceptible individual and the expression of the resulting outcome (infection versus no infection) is short. Measurement of the early immune responses elicited against bacterial invasion may elucidate not only the role of individual factors but also whether these factors act together. Such analyses should differentiate between bacterial invasion (the initial event) and the outcome, which may be dichotomous as either a bacterial infection or an immunity-mediated protection (no infection). These concepts are applicable to bovine intra-mammary infections, also known as infectious mastitis, which is a group of diseases that are of major economic importance to the dairy industry. The major cause of bovine mastitis is infection of the udder by pathogenic bacteria. A wide variety of bacteria can be involved, but the most common mastitis pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, *S. dysgalactiae*, and *S. uberis*. *Staph. aureus* is one of the most frequently found etiological agents of bovine subclinical mastitis (2). Now, the two central tools that are used to monitor bovine intra-mammary infections are the somatic cell count (SCC) and bacteriologic tests of milk. Both of these tools assess only the outcomes (end results) and not the processes (intermediary instances). In the mammary gland, the number and distribution of leukocytes are important determinants of whether the udder can successfully defend against pathogens. Most studies have addressed the distribution of somatic cells in the milk, but leukocytes in the udder tissue could differ from those found in the milk and may play a more important role in mammary immunity. Moreover, a somatic cell count of milk is a total cell count and not a differential leukocyte count. In addition, there are no cut-off values for mastitis versus non-inflammatory mammary glands (11). The immune response in bovine milk infected with various pathogens can more exactly evaluate bovine mastitis. Indeed, leukocyte surface markers of CD4, CD8 and CD11b are critical molecules that have an influence on antibacterial responses (9-11). We chose to study the leukocyte surface markers CD11b, CD4 and CD8 and investigated the differences in expression level of markers between infected/non-infected groups. The aim of this study was to determine: 1) the pathogen-induced mastitis indices, 2) the relationship between the somatic cell count and leukocyte antigen surface markers, and 3) the correlation between simple infection and co-infection.

[1]Corresponding author.
E-mail : jpark@jbnu.ac.kr

Materials and Methods

Milk samples

A total of 126 sample isolates from 15 milking farms in Jeonbuk were investigated. The herds were from different geographical locations in Jeonbuk. Somatic cell counts were obtained using a Coulter Counter Model Z1 (Coulter Electronics Ltd., Beds., England) according to the revised protocol of the A2B sub-group of the International Dairy Federation (IDF) [Mastitis Experts; IDF (1991)]. Milk samples were kept at 4°C and processed within 24 hrs after the sample was collected. Bacterial cultures were also performed in Tryptic Soy Broth (TSB, Difco laboratories) at 37°C for approximately 17 hrs.

Sample preparation for flow cytometry analysis

The volume of milk containing total number of 1×10^6 cells was divided into 30 mL test tubes, one tube for each

Abs and the control. The tubes were centrifuged for 15 min (400 × g), and then the fat was removed by aspiration and the supernatant was discarded. The pellets were re-suspended in phosphate-buffered saline (PBS) and washed by centrifugation once more for 10 min (400 × g). To each tube containing cell pellets, 10 μL of one of the Abs or PBS (as a negative control) was then added and mixed gently and then allowed to incubate for 1 h at 4°C. Cells were washed in PBS and 10 μL of one (single) or two (double) of the conjugated antibodies were added, mixed gently, and incubated for an additional 30 min at 4°C. Following incubation, the cells were washed and re-suspended in PBS to a final volume of 500 μL, and a total of 10,000 events were read.

Flow cytometry analysis

The primary antibody used for the detection of polymorphonuclear cells (PMN) or monocytes was an antibody against CD11b (MCA 1777S; SEROTEC, Duesseldorf, Germany). The antibodies for bovine CD4 (MCA1653S; SEROTEC, Duesseldorf, Germany) and CD8 (MCA837PE; SEROTEC, Duesseldorf, Germany) were used to detect lymphocytes in the samples. The secondary antibody for CD11b was rabbit F (ab`)2 anti-mouse IgG (H + L) that was conjugated with R. phycoerythrin (STAR12A; SEROTEC, Duesseldorf, Germany).

The cells were analyzed using a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometry, equipped with a 15 mW air-cooled argon laser. Leukocytes were identified and quantified based on forward and side scatter parameters. The antigen surface density of the cells was calculated from the percentage of cell that stained with fluoresces of antibody. A gate which defined 95% of the unstained control samples as negative was used to define percent positive cells (Fig 1). Data analysis including spectral compensation was performed using FCS Express (FCS Express Version 3, De Novo Software).

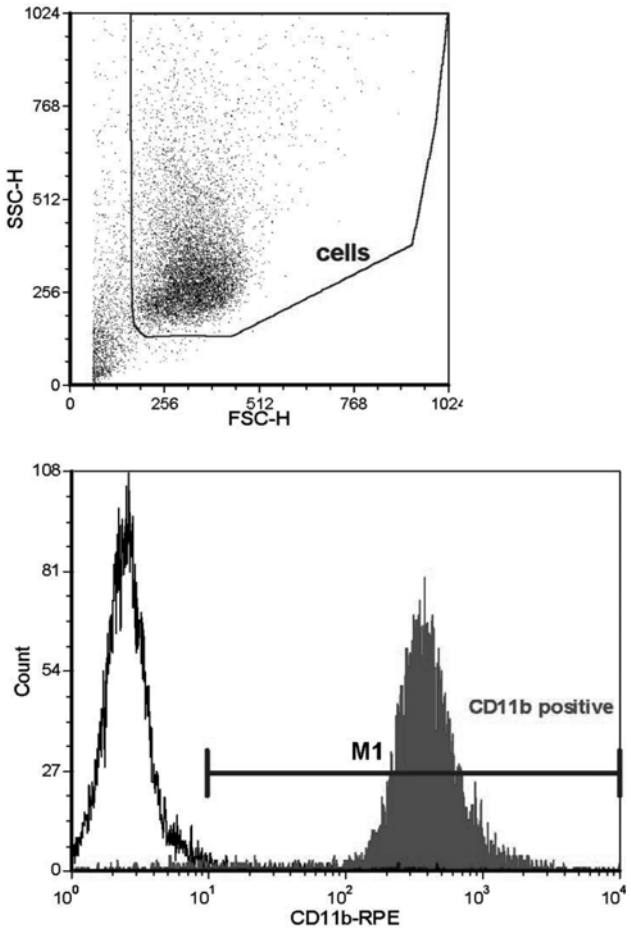

Fig 1. Flow cytometric analysis of a leukocyte marker of CD11b in bovine milk. Cells were isolated from the milk by centrifuge and incubated with monoclonal antibody against CD11b for 30 min at 4°C. Debris were excluded by forward and 90°C side scatter, and the percent CD11b positive cells were scored by gating M1 region which includes less than 5% of the unstained cells in the histogram.

Pathogen identification

Pathogen isolates were studied after they grew as pure cultures overnight, and the genomic DNA was extracted according to the protocol for gram positive bacteria used GENE ALL™ (GeneAll Bio Ststem, Seoul, Korea) kit. PCR for targeting 16S or 23S rRNA of *Staphylococcus aureus*, *Streptococcus agalactiae, S. dysgalactiae, S. uberis* was performed as previously described (7).

Statistical analysis

Data analysis was performed with the Statistic Package for Social Scientists for Windows Release 15.0.0 (SPSS, LEAD technologies, Inc.). All results were explored and tested data normality by Shapiro-Wilk test. Differences between the non-infected and infected group were calculated by using Student's *t*-test (parametric) or Mann-Whitney U test (non-parametric) for independent group. Comparison of immunological markers in various pathogen-infected samples was analyzed by one-way ANOVA test (parametric) or Kruskal-Wallis test (non-parametric). For the post hoc test, the Scheffe test was used, and the Bonferroni correction was applied. Correlations with immunological indices and pathogens were evaluated by Pearson's correlation test. A p value < 0.05 was considered significant.

Results

Bacterial culture

Out of the 126 milk samples tested, 65 samples (50.8%) were positive for *Staph. aureus*, 4 samples (3.2%) were positive for *S. agalactiae*, 74 samples (57.9%) were positive for *S. dysagalactiae*, and 37 samples (29.4%) were positive for *S. uberis*. Staphylococcus co-infection occurred in 61.8% percent of the total bovine mastitis samples. In the infectious group, the percentages of the samples that were single pathogen-infected, double pathogen-infected, and triple-pathogen infected were 45.1%, 38.2% and 16.7%, respectively.

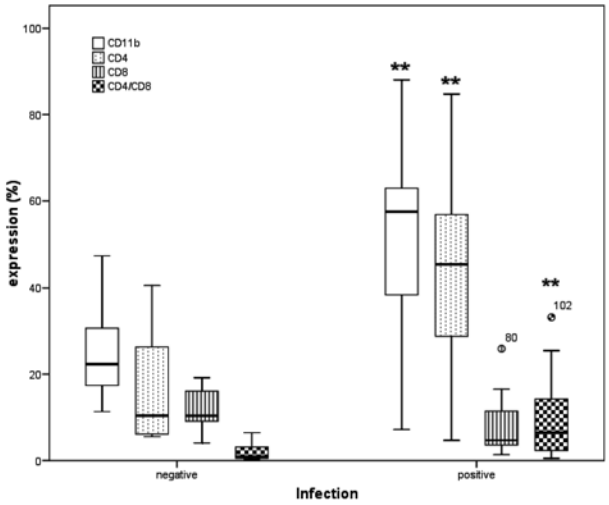

Fig 2. The variation in leukocyte surface markers between pathogen-infected and pathogen-free milk (**$p < 0.01$ vs non-infected sample). The box plot present as median values (line), IQR (boxes), and 5th to 95th percentiles (whiskers).

Variation immunological markers between pathogen-free and pathogen-infected milk

The expression levels of CD11b and CD4 in the infectious group were significantly greater than the non-infectious group (Student-*t* test, $p = 0.003$, $p = 0.002$, respectively). In addition, the CD4/8 expression ratio in the infectious group was significantly greater than that of the non-infectious group (Mann-Whitney U test, $p = 0.002$) (Fig 2). The comparison of the immunological variables among group infected with different pathogens yielded no significant differences. However, the percentages of CD11b and CD4, CD4/8 expression were positively correlated with the number of infectious pathogens present ($p = 0.006$, $p = 0.006$ and $p = 0.021$, respectively) (Table 1).

The correlation between SCC and immunological indices

When somatic cell counts and immunological indices were analyzed, the CD4/8 percent ratio and somatic cell count were positively correlated (r = 0.744). The percentage of CD11b was also correlated with the percentage of CD4 (r = 0.501, 0.416).

Discussion

The two central tools used to monitor bovine mammary infections are SCC and bacteriologic tests. These tests are based on the end results and are not able to detect intermediary instances. Some *S. aureus* strains that are isolated from both symptomatic and asymptomatic individuals are indistinguishable (1). Furthermore, the SCC is a total cell count and not a differential leukocyte count.

CD11b is major component of granulocytes in milk (8). A recent study revealed that CD11b was significantly different between SCC < 100×10^3/mL and SCC ≥ 100×10^3/mL. However, the CD3 and CD4 counts were not significantly different between SCC < 100×10^3/ml and SCC ≥ 100×10^3/mL (3). In the present study, the expression level of CD11b in the pathogen-infected milk samples was significantly increased from the non-infected milk samples, and it was also increased in co-infection, especially infected with two species. It was probably the proportion of leukocytes was significantly higher in all of the infected quarters in comparison to those free of infection, which might be more specific marker than SCC.

Table 1. The percentage (mean ± S.E.M) of leukocytes displaying specific markers according to the number of pathogen species present in the milk (**$p < 0.01$, *$p < 0.05$ among groups according to the number of pathogen species, §$p < 0.0125$ vs noninfection in post hoc test of Bonferroni correction).

Infection	Percentage of positive cells			
	CD11b**	CD4**	CD8	CD4/CD8*
Non-infected	25.3 ± 4.8	15.1 ± 5.0	12.9 ± 1.6	1.3 ± 0.5
Infected (1 pathogen)	42.6 ± 6.7	35.3 ± 7.3	8.0 ± 1.9	9.4 ± 3.8
Infected (2 pathogens)	58.1 ± 6.0§	51.0 ± 7.4§	7.2 ± 1.1	10.1 ± 2.5§
Infected (3 pathogens)	59.7 ± 0.2	53.7 ± 3.0	3.7 ± 0.7	15.0 ± 3.5

In addition, the mean CD4/8 ratio was significantly greater to 9.30 ± 2.84 ($p < 0.01$) in the pathogen-infected milk samples whereas 1.97 ± 0.85 in the non-infected milk samples in this study. In a previous study, the CD8 percentages in the milk of cows with mastitis have been reported to be elevated (6). An increased percentage of bovine milk CD4 cells had been also reported in animals infected with *S. uberis*, *E. coli*, and *Staph. aureus* (12). The mean CD4 percentages were also significantly higher to 45.3% compared to the non-infectious group in this study. However, in the present study, the mean CD8 percentages did not differ significantly. This study was conducted with spontaneous infections, and thus we could not determine whether CD8 cells are present in the early or late immune-inflammatory phases. Because lymphocytes bearing CD8 are the predominant milk T-cell subset in healthy cows (5,13), an increased CD4/8 ratio suggests a shift in the phenotype profile in response to infection. Furthermore, the CD4/8 ratio is usually less than 1 in a healthy mammary gland (4). However, in this study, no differences in leukocyte marker values were found among the three major pathogens (*Staph. aureus, S. dysgalactiae,* and *S. uberis*) identified by PCR analysis.

The percentage of CD11b and CD4 in the sample was significantly correlated with the number of infecting pathogens ($p < 0.05$). As results of this study, these findings indicated that the percentages of leukocytes expressing CD11b as well as the CD4/CD8 ratio can be applied to diagnose bovine mastitis. The CD 4/8 ratio in particular varied according to the presence and the number of infections, though it did not appear to be dependent on any one particular pathogen.

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우유의 체세포내 면역 표지자 분석을 통한 소 유방염 진단

유도현 · 이종현 · 송루희 · 노동호 · 이영화 · 이미진 · 박진호[1]

전북대학교 수의과대학

요 약 : 본 연구는 소의 유선 내 감염(유방염)과 체세포 면역 표지자의 상관 관계 분석을 위하여 자연적으로 감염된 젖소와 건강한 젖소의 우유를 비교한 단면조사 연구이다. 유방염에 이환된 31마리의 국내 젖소의 우유에서 세 가지의 체세포 면역 표지자(CD11b, CD4, CD8)의 발현과 체세포 수(SCC), 그리고 세균학적인 분석(배양 및 PCR검사)을 통한 감염 여부 및 병원체의 종류에 대하여 분석하였다. 그 결과 감염된 젖소의 우유는 건강한 젖소의 우유보다 체세포 내의 CD11b와 CD4발현이 유의적으로 증가하였으며, CD4/CD8비율도 높았다. 그러나, 병원체의 종류에 따른 증가된 체세포 면역 표지자와는 커다란 연관성을 보이지 않았으나, 감염된 병원체의 수와 관련해서는, 체세포 면역 표지 인자 CD11b, CD4의 발현 그리고 CD4/8 비율의 증가와 현저한 관련이 있었다. 이러한 연구 결과로 볼 때, 면역 표지자를 이용한 우유의 체세포 분석을 통하여 특발성 소 유방염의 진단 및 관리에 유용하게 활용될 수 있을 것이다.

주요어 : 소 유방염, 체세포 분석, 면역 표지자(CD11b, CD4, CD8).