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Milk Leucocyte Populations in Heifers Free of Udder Infection

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With 2 tables and 1 figure

Summary

Improvement of udder health through a process of genetic selection is related to heritability and the role of the specific trait in the probability of an individual cow developing an infection. It was suggested that different patterns of leucocyte population of the healthy gland are a significant factor in mastitis. Thus, in order to analyse the heritability of a trait and its correlation with udder health, the present study examined the leucocyte populations of uninfected mammary glands, their variability among quarters in a particular cow, and the changes that occur during lactation. Each one of the 20 cows examined was tested on average 3.06 times during lactation. The somatic cell count (SCC)/ml ranged from 12 000 to 151 000, the coefficients of determination (R^2) were higher than 0.5 for SCC. No significant differences were found in the dependent variables between the sampled times (test) nor any interaction between the slopes calculated for the cows over time. No significant differences were found among quarters within a cow for any of the dependent variables including SCC. The effect of the cow trait was found to be significant for polymorphonuclear (PMN), macrophage (M∅), and T-lymphocyte-bearing CD4^{+}. The number of lymphocytes labelled with the anti-B monoclonal antibodies was negligible. In conclusion the patterns of leucocyte populations in milk together with the variance among cows should enable an analysis of the heritability of this trait and its correlation with udder health in a future study.

Introduction

The inflammatory response reflects the immunological response of the mammary glands against invading pathogens and other antigenic factors. The resident cell population of the healthy gland initiates the immune response following pathogen penetration through the teat canal. These cells initiate the inflammatory response and together with the newly recruited leucocytes are necessary to eliminate the invading bacteria (Sordillo et al., 1997). Previous reports have shown that numbers as well as functionality of leucocytes, mainly neutrophils, are crucial for the elimination of new intramammary infection (Hill, 1981; Grommers et al., 1989). The process of migration from the bloodstream into the contaminated udder tissue involves activation of both neutrophils and the post-capillary venular endothelium (Dustin et al., 1986; Rothlein et al., 1986; Staunton et al., 1988). Thus, an effective mammary gland response to a microbial agent is time dependent and also depends upon the type of bacteria and the resident cell population. In uninfected, normal mammary glands there is variation in the total somatic cell count (SCC) with macrophages (M∅) being the predominant cell type, followed by lymphocyte, polymorphonuclear (PMN) and epithelial cells (Concha et al., 1986; Miller et al., 1990, 1991, 1993).

Improvement of udder health through the process of genetic selection can be achieved by a direct approach such as decreasing the incidence of clinical mastitis or by an indirect one such

as selection for marker traits related or positively correlated with udder health. Changes in genetic potential are affected by genetic variability in the population, accuracy in the identification of animals with superior genetic potential, intensity of the selection process and interval between successive generations. Leslie (1995) in his review *Genetic selection for resistance to mastitis* described four types of traits which have been proposed as the basis for genetic improvement of udder health: (1) clinical mastitis incidence, (2) conformational traits related to udder health; (3) SCC; and (4) major histocompatibility complex and other markers of immune function.

The heritability of SCC is moderate (0.13) (Weller and Ezra, 1997). However, there are no reports to our knowledge on the heritability of the milk leucocyte population patterns in healthy animals.

There is a high probability that leucocyte population patterns of the uninfected mammary gland are a trait related with high probability to udder health. In order to perform a selection scheme it is important to know the heritability and its repeatability in the milk before infection occurs. Based on the variability of leucocyte populations in the uninfected mammary gland and the low to moderate heritability of SCC, different leucocyte patterns have been suggested to play an important role in the probability of an individual gland or cow developing an infection. The aim of the present study was to examine the leucocyte populations of uninfected mammary glands, their variability among quarters of a particular cow, and the changes that occur during lactation.

Materials and Methods

Animals

Israeli Holstein cows in their first lactation were selected from two commercial herds. A total of 20 cows were chosen according to bacteriological examinations of quarter milk samples taken on three consecutive days. Each of the cows had at least two quarters classified as free of bacteriology contamination. Cows were kept on a slatted floor providing $10\,m^2$ shade per cow and a $10\,m^2$ area of dirt yard per cow, and were milked three times daily by a computerized milking system (S.A.E.; Afikim, Israel) in which cows were identified automatically and composite milk yield, its electrical resistance, and milking flow times were registered at every milking. Food was offered in mangers located in the sheds.

Study layout

Cows were first tested 100–120 days after calving and every 50–60 days thereafter until drying off (i.e. three to four times). During the morning milking, the tested quarters were cleaned with tap water, dried with individual towels, disinfected with an individual non-woven towelette moisted with chlorhexidine, cetrimide and ethanol (Medi-Wipes, AL Baad Messuot Itzhak, Israel) and the first three milk squirts discarded. For bacteriological testing, approximately 5 ml of milk were taken into a sterile tube. Foremilk (50 ml) was taken at the same time from each quarter to determine the SCC and the differential cell count. A quarter that was found to be contaminated during the course of the experiment was included in the analysis only until that point in time and disregarded thereafter.

Bacteriological examinations

Duplicate quarter foremilk samples were taken aseptically according to IDF (1981) procedures and submitted to the laboratory for bacteriological analysis which was performed according to the National Mastitis Council protocols (Barnes-Pallesen et al., 1987). A volume of 0.01 ml of each milk sample was spread over blood-agar plates (Bacto-Agar; Difco Laboratories, Detroit, MI) containing 5% of washed sheep red blood cells, and into MacConkey agar plate (Difco). All plates were incubated at 37°C and examined for growth at 18 and at 42 h.

Antibodies and conjugates

The monoclonal antibodies (mAbs) (VMRD Inc. Pullman, WA, USA) used for the detection of different leucocytes were: (1) anti-lymphocyte; anti CD4^{+}: CACT 138A; anti CD8^{+}: CACT 80C and anti B: GB25A and BAS9A, all diluted 1 : 500; (2) anti-monocyte/macrophage; CH137 and BAQ151A, diluted

1 : 200; (3) anti-granulocyte; CH138A and MM20A, diluted 1 : 100. All mAbs were species-reactive with bovine cells.

The polyclonal antibody used was goat F(ab′)2 anti-mouse IgG (H+L) conjugated with fluorescein isothiocyanate (FITC) that exhibited minimal cross-reaction to human-, bovine- and horse-serum proteins (Jackson Immuno Research Lab., West Grove, PA, USA).

Laboratory procedures

Milk samples were kept on ice for about 2 h, before processing. SCC was determined with a Coulter cell counter Z1 model (Coulter Electronics Limited, Luton, England). Leucocyte differential counts were carried out by flow cytometry (FACS-can, Becton Dickinson, San Jose, CA, USA).

Differential leucocyte count

Milk samples were kept at room temperature and analysed not more than 3 h after collection. To assess the different leucocyte populations in each sample, a volume of 10 ml of milk containing about 5×10^5–1×10^6 cells was distributed into 15 ml test tubes, one tube for each mAb and the control. The tubes were centrifuged for 10 min (230 g, 4°C), the fat was removed by aspiration, the supernatant was discarded and the pellets were resuspended in 15 ml phosphate-buffered saline (PBS) and washed by centrifugation once more for 5 min (200 g, 4°C). To each tube, 50 μl of one of the mAbs or PBS (as a negative control) was added, mixed gently and incubated for 1 h at 4°C. Cells were washed three times in PBS and 50 μl of the conjugated antibody was added, mixed gently and incubated for 30 min at 4°C. Following incubation the cells were washed and resuspended in PBS to a final volume of 1 ml. To calculate the percentages of the different leucocytes, 10 000 events were read per sample. The cursor was set in a way that only 2% of the events in the control were to the right of it. In samples labelled with mAb, the percentage of events to the right of the cursor was considered positive. The absolute cell number for each cell type was calculated from the SCC determined by the cell counter. In addition, total lymphocytes was evaluated by gating the cells on a dot plot with forward-angle light scatter by side-light scatter map.

Statistical analysis

Data was analysed using the SAS general linear model (GLM) procedure (SAS/STAT® User's Guide, 1990). Dependent variables were SCC and SCC transformed to $\log_2$ (LCSS), absolute numbers of different leucocytes transformed to $\log_2$: PMN, M∅, total lymphocytes (gate) and T-lymphocytes bearing CD4^{+} or CD8^{+}. The effects on the dependent variables and the independent traits: herd, season, cow, quarter, days in milk and days in milk squared were examined with the model:

$$Y_{ijkl} = H_i + S_j + C_k + a_1 * D + a_2 * D^2 + a_3 * D * C_k(H) + QU_{ijkl}(H_i * C_{kl}) + e_{ijkl}$$

where: Y = dependent variable; H = herd, $i = 1, 2$; S = season, $j = 1, 2$: $j = 1$ from January to June, otherwise $j = 2$; C = cow, k in herd i, $k = 1, \ldots, 20$; QU = udder quarter l in cow k in herd i, $l = 1, \ldots, 4$; D = days in milk, from calving to the test day; a_1, a_2, a_3 = regression coefficients; e = error. The results are given in $\log_2$ of the cell number.

Results

Results showing the different leucocyte populations in the milk from an udder that was free of contamination with the same SCC are presented in Fig. 1. Fig. 1a–c demonstrates a pattern of high PMN cells, mainly neutrophils and low lymphocyte numbers. In Fig. 1d–f, a pattern of high lymphocytes (CD4^{+} and CD8^{+}) and low PMN can be observed.

Means, minimum and maximum values of the dependent variables are summarized in Table 1. The variances in total lymphocytes and T-lymphocytes bearing CD4^{+} or CD8^{+}, M∅, and PMN were higher than LSCC, i.e. the differences between the minimum and maximum value of LSCC were smaller. The total SCC variation was 28 000 cells, i.e. 99% of the cows had between 12 000 nd 151 000 cells. The coefficients of determination (R^2) were higher than 0.5 for SCC and LSCC, and higher than 0.6 for the leucocytes tested (Table 2). The interaction between days in milk and cow ($D * C$) estimates for the slopes between cows over the traits was not significant for most traits, supporting the assumption that all cows had the same slope (Table 2), that is, no significant difference was found between the sampling time (test) in a cow.
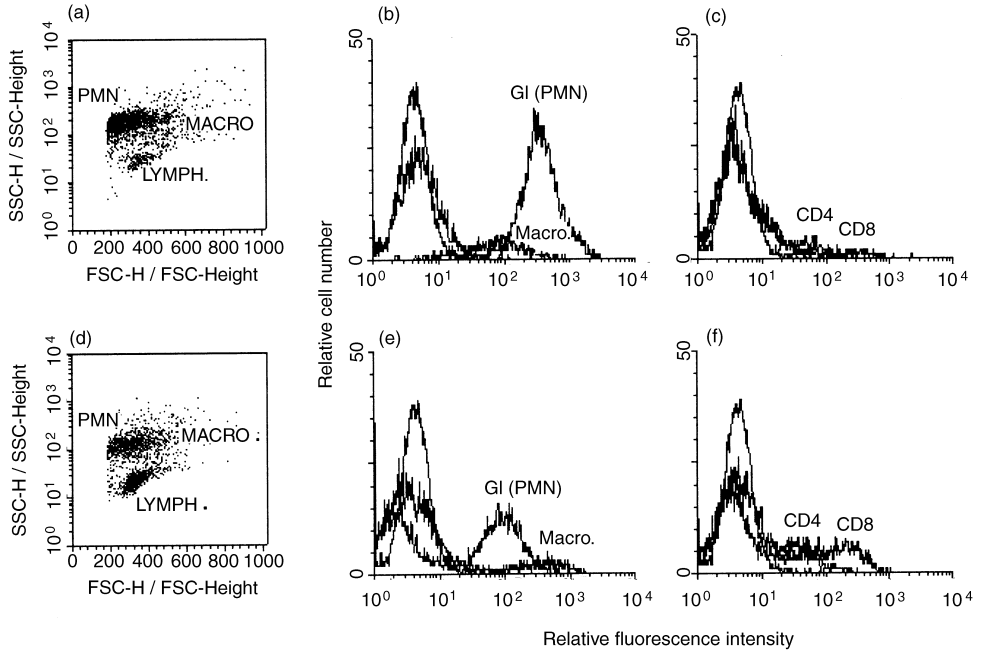
Fig. 1. Dot plot of forward-angle light scatter (FSC) versus logarithm side-light scatter (SSC) map of milk leucocyte (a, d) and histogram fluorescence analysis of milk leucocytes (b, c and e, f) from two uninfected cows. (a), (b) and (c) demonstrate a pattern of high polymorphonuclear (PMN) cells, mainly neutrophils and low lymphocyte numbers and (d), (e) and (f) a pattern of high lymphocytes (CD4$^+$ and CD8$^+$) and low neutrophils.

Table 1. Means $\pm$ SD, minimum and maximum of the dependent variables: SCC and log$_2$ (SCC) (LSCC), total lymphocytes and lymphocytes bearing CD^{4+} or CD^{8+}, macrophages (M$\varnothing$) and polymorphonuclear cells (PMN) of 50 quarters free of infection from 20 cows tested two to four times during their first lactation

Variables	Mean $\pm$ SD	Minimum	Maximum
Quarters	2.5	2	4
Test	3.06	2	4
SCC ($\times 10^3$)	50 $\pm$ 28	12	151
LSCC	5.5 $\pm$ 0.7	3.6	7.2
Total lymphocytes	3.1 $\pm$ 0.9	0.8	6.1
CD^{4+}	0.8 $\pm$ 1.2	-2.2	4.1
CD^{8+}	2.0 $\pm$ 1.0	-0.6	5.1
M$\varnothing$	1.8 $\pm$ 1.2	-0.97	4.8
PMN	2.9 $\pm$ 1.4	-0.2	6.3

The results are given in log$_2$ of the cells number.

Each cow was tested on average 3.06 times during lactation (Table 1). No significant differences were found in the independent traits: herd, season and days in milk on most of the dependent variables. No significant differences were found among quarters within a cow for any of the dependent variables including SCC (Table 2). The effect of the cow trait was found to be

Table 2. The *P*-value effects of the independent variables: cow (*C*), quarter (*QU*), days in milk (*D*) and days in milk * cow (*D**C), and of the dependent variables: SCC and LSCC, total lymphocytes and lymphocytes bearing CD^{4+} or CD^{8+}, macrophages ($M\emptyset$), and polymorphonuclear cells (PMN), of 50 quarters free of infection from 20 cows tested three to four times during their first lactation

Trait Variables	*D*	*C*	*D**C	*QU*	R^2
SCC	NS	NS	NS	NS	0.54
LSCC	NS	NS	NS	NS	0.53
Total lymphocytes	NS	NS	NS	NS	0.65
CD^{4+}	NS	0.03	NS	NS	0.66
CD^{8+}	0.01	NS	NS	NS	0.64
$M\emptyset$	NS	0.006	0.007	NS	0.68
PMN	NS	0.05	NS	NS	0.64

NS, not significant.

significant for PMN, $M\emptyset$, and T-lymphocytes bearing $CD4^+$. The number of lymphocytes labelled with the anti-B mAb was negligible (data not shown).

Discussion

Any method for cell differentiation (light microscopy or flow cytometry) that includes milk skimming could to some extent distort the actual leucocyte population pattern, due to attachment of cells to the fat globules which were removed before analysing. The low variability found between the tests (time of sampling) and quarter for most of the leucocytes tested indicated that an equivalent percentage loss was detected on subsequent examination. This result confirms the study of Dulin et al. (1982) which showed no significant differences between the direct smear (whole milk) and the cytospin procedures (defatted milk) when using light microscopy. It is therefore to be concluded that the differential cell counts in milk are accurate to a high extent.

The low variability in SCC between and within cows during lactation enabled an analysis of the leucocyte patterns in the milk without statistical manipulations, despite the low number of cows. The similarity found among quarters within a cow for the SCC and the different leucocytes, and the high repeatability in a particular cow during the lactation period, suggests that the milk leucocyte pattern in uninfected, normal mammary glands is genetically controlled. There is a high probability that leucocyte patterns in milk are traits related to udder health. However, it is not known whether the heritability of this trait is positively correlated with udder health. Thus, the method used to define the leucocyte pattern in milk together with the variance among cows, should enable an analysis of the heritability of this trait and its correlation with udder health.

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