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Alteration in the activity of blood and milk leukocytes together with the serum enzyme profile during sub-clinical mastitis in cross-bred cows

SWARNAVA GAIN[1], JOYDIP MUKHERJEE[2], SAIBAL CHATTERJEE[3], SUBHASHIS BATABYAL[4] and CHANCHAL GUHA[5]

West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal 700 037 India

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ABSTRACT

In vitro activity of blood and milk leukocytes together with serum enzyme profile during sub-clinical mastitis in crossbred cows were evaluated after collection of blood and milk samples from normal (10) and sub-clinical mastitic (10) cows. Blood total leukocyte counts (TLC) and differential leukocyte counts (DLC) were estimated by standard hematological procedure. Milk somatic cell counts (SCC) was performed microscopically. *In vitro* phagocytic activity of blood and milk neutrophils was performed by colorimetric nitro blue tetrazolium (NBT) assy and mitogen concanavalin A (con A) induced blood and milk lymphocyte blastogenic response was evaluated by colorimetric MTT (tetrazolium) assay. Serum total protein and alkaline phosphatase (ALP) were measured by the stand biochemical methods. The alanine amino transferase (ALT) and aspertate amino transferase (AST) activities in serum were estimated by commercially available kit. Milk SCC was significantly higher in sub-clinical mastitic cows. Phagocytic index of both blood and milk neutrophils was significantly lower in sub-clinical mastitic cows than normal animals. Con- A induced blood and milk lymphocyte blastogenic response was significantly lowered in sub-clinical mastitic cows than. Serum albumin, globulin ratio decreased significantly during sub-clinical mastitis. Serum AST and ALP level in sub-clinical mastitic cows was significantly higher. The study indicated decreased blood and milk leukocyte activity and higher AST and ALP during the sub-clinical mastitis which could be used as a diagnostic tool for sub-clinical mastitis.

Key words: Leukocytes, Immune activity (neutrophils, lymohocytes), Sub-clinical Mastitis, Serum enzyme

Sub-clinical mastitis is a great challenge among the dairy farmers as it is subtle and more difficult to detect. It is characterised by reduced host defence mechanism (Owen *et al*. 2000) as well as alterations in some enzymes and metabolites (Ibtisam El Zubeir, 2005, Matei *et al*. 2010, Andrei *et al*. 2009) The purpose of the the present investigation was to evaluate the in vitro phagocytic activity of neutrophil and mitogen induced lymphocyte blastogenic response (both blood and milk) in relation to the alteration in serum enzyme activity during sub-clinical mastitis in cross breed cows.

MATERIALS AND METHODS

Selection of experimental animals and sampling: Crossbred cows (normal, 10; sub-clinical mastitic, 10) of third-fourth parity were selected from the local farms. All the animals were under early lactation (70–100 day) and the average yield per animals was 6.5±1.1 and 3.2±0.65 lit/ day, respectively, for normal and infected cows. All the cows were maintained in loose housing system with brick flooring and managed as per the practices followed in the farm. They were offered ad lib. green fodder and calculated amount of concentrate mixture based on milk production. Freshwater was available ad lib. at all the times of the day. Animals with sub-clinical mastitis were obtained after routine evaluation by bromothymol blue (BTB) card test (Chanda et al. 1989) and modified California mastitis test by commercially available kit as per manufacturer's protocol.

Blood (15 ml/animal) was drawn from all the animals in sterile heparinised vacutainer tube from jugular vein puncture, posing minimum disturbance to the animal during collection on the same day of milk sampling. Samples of composite milk (200 ml/animal) (representing all quarters) were collected into sterile tubes and immediately transported to laboratory in ice for further processing.

Blood sample collected was allowed to clot at 25ºC and the serum was collected in a sterile vial. Then the serum sample was further centrifuged at 5,000 rpm/ 5 min, to

Present address: [1]M.V.Sc. Scholar (swarnavagain1989 @gmail.com), Department of Veterinary Biochemistry, [2]Assistant Professor (joyphy@gmail.com), Department of Veterinary Physiology, [3]Reader (dr.schattopadhyay@gmail.com), [4]Reader and Head (batabyals2009@gmail.com), Department of Veterinary Biochemistry, [5]Professor (profcguha@gmail.com), Department of Veterinary Epidemiology and Preventive Medicine.

remove residual RBC and it was stored at –25ºC for further estimation.

Estimation of total leukocyte count (TLC) and milk somatic cell count (SCC): TLC was enumerated by hemocytometer as per standard hematological procedure as described by Schalm *et al.* (1975) and expressed in term of thousands per cubic millimeter (Cmm). Somatic cell counts of milk samples were measured microscopically by the method of Dang *et al*. (2008).

Isolation of neutrophils and lymphocytes from blood and milk: Isolation of lymphocytes from blood was carried out by density gradient centrifugation with lymphocyte separation medium as per manufacturer's protocol. Isolation of neutrophils from peripheral blood was performed using hypotonic lysis of erythrocytes as described by Mehrzad *et al*. (2002).

Isolation of polymorphonuclear neutrophils from milk was performed by the method of Mehrzad *et al*. (2002) within 2 h of sample collection. Isolation of lymphocytes from milk was done by density gradient centrifugations as described by Mukherjee and Dang (2011) and Mukherjee *et al*. (2013).

Viability of isolated blood and milk leukocytes: The viability of all milk leukocytes was determined using Trypan Blue exclusion test. The viability of blood and milk lymphocytes in different experiments was found to range between 95–98% and 80–85%, respectively, within 6 h of processing and declined gradually afterwards. The viability of blood and milk neutrophils was around 90–93% and 80–83% after washing and declined afterwards.

Estimation of in vitro phagocytic activity: After isolation, in vitro phagocytic activity of blood and milk neutrophils were determined by colorimetric NBT assay described by Chai *et al.* (2005).

Estimation of in vitro lymphocyte proliferation response: The isolated milk lymphocytes were allowed to proliferate with and without mitogen [Concanavalin A (Con A)] to determine the difference between cell proliferations. Con A was taken as it stimulates T-lymphocyte. The proliferative response of lymphocyte was estimated using the colorimetric MTT (tetrazolium) assay according to the procedure given by Mosmann (1983).

Estimation of serum total protein: Serum total protein in each sample was determined by Biuret method of Reinhold (1953) in a photoelectric colorimeter using a yellow green filter. The total protein value was expressed as g/dl.

Estimation of serum alkaline phosphatase, serum alanine amino transferase (ALT) and aspertate amino transferase (AST): The serum alkaline phosphatase was measured by method using 4-amino antipyrine, described by Tietz (1999) and expressed as U/litre. The ALT and AST activities in serum were estimated according to the manufacturer's institution of commercially available kit using 2,4-Dinitrophenyl hydrazine (DNPH).

Statistical analysis: All analysis was done using SYSTAT software package. Data from different experiments are presented as mean±SE. Significance of all the parameters except, phagocytic index of neutrophils and stimulation index of lymphocytes were analysed by t-test. Significance of phagocytic index of neutrophils and stimulation index of lymphocytes was tested by employing two way ANOVA considering group (normal and mastitic) and source (blood and milk) as factors by SYSTAT software package.

Statistical model for two way ANOVA

$$Yij = \mu + G_{ij} + D_{ij} + (GD)_{ij} + e_{ij}.$$

Where, μ, population mean; G_{ij}, effect of group (normal and subclinical mastitic), D_{ij}, effect of source (blood and milk); $(GD)_{ij}$, interaction; and e_{ij}, random error.

RESULTS AND DISCUSSION

Blood TLC, DLC and milk SCC in normal and mastitic cows: The TLC (× 10^3/μl) was higher in sub-clinical mastitic than normal cows but, the difference was non significant (Fig. 1; Table 1).

There was no significant alteration in DLC between normal and sub-clinical mastitic cows. However, the eosinophil percentage was nonsignificantly higher in sub-clinical mastitic cows whereas lymphocyte percentage was lower.

Milk SCC was significantly (P<0.01) higher in sub-clinical mastitic cows than normal. There was about 3- fold increase in milk SCC during sub-clinical mastitis.

The TLC lavel in this study was normal as reported by

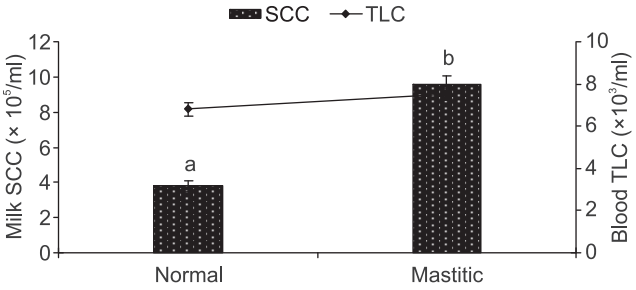

Fig. 1. Milk SCC and blood TEC in normal and mastitic cross-bred cows ; Values are expressed as mean±SEM and the values which lacks a common letter are significantly (P<0.01) different.

Table 1. Blood DLC, serum total protein and enzymes in normal and mastitic cross-bred cows

Parameters	Normal	Mastitic
Neutrophils (%)	25.0±3.1	25.0±2.1
Eosinophils (%)	6.0±0.5	7.2±0.5
Basophils (%)	ND	ND
Monocytes (%)	4.0±0.2	3.6±0.1
Lymphocytes (%)	65±2.7	64±2.1
Total protein (g/dl)	6.98±0.31	6.85±0.22
Albumin:Globulin	1.39^a±0.03	0.40^b±0.02
Serum AST (U/L)	85.2^a±6.80	114.5^b±8.10
Serum ALT (U/L)	21.7±3.80	25.1±2.10
Serum ALP (U/L)	36.5^a±2.00	53.4^b±3.40

Values are expressed as mean±SEM and the values within a row which lacks a common letter are significantly (P<0.01) different.

Jain (1986). In the current study, there was no significant alteration in the TLC in normal and mastitic cows, which is in accordance with the observation of Zaki *et al*. (2010). However some studies reported increased leukocyte counts in mastitis which may cause an inflammatory reaction that results in the elimination of infection and also tissue injury that may lead to fibrosis in mammary function (Latimer *et al*. 2003). Kremer et al. (1993) reported that before and during experimental mastitis, the chemotactic response and the number of circulating polymorphonuclear leukocytes were greater. Fakhar-uz-Zaman *et al*. (2009) indicated an increase of neutrophils and decrease of lymphocytes and monocytes, whereas, no significant difference was seen for eosinophils and basophils during sub-clinical mastitis in buffaloes. The lack of significant differences in this study could be attributed to the phase and progression of the disease.

Milk SCC is one of the commonly used tools to monitor udder health and thus milk quality (Kehrli and Shuster 1994). The SCC found in this investigation was similar to the reports by Dang *et al*. (2008). Grommers *et al*. (1989) reported that the severity and duration of mastitis is critically related to the promptness of the leukocyte migratory response and the bactericidal activity of cells at the site of infection. However, Kehrli and Shuster (1994) stated that high SCCs in milk are not the cause of mastitis; instead, they are a necessary and correlated response to combat microbes in the mammary gland.

Phagocytic index of blood and milk neutrophils in normal and mastitic cows

The phagocytic index is significantly (P<0.001) higher in blood than milk neutrophils in both normal and sub-clinical mastitic condition (Fig. 2). Phagocytic index of both blood and milk neutrophils was significantly (P<0.05) lower in sub-clinical mastitic cows than normal animals.

Mammary neutrophils or polymorphonuclear cells (PMN) provide the first line of defense against invading mastitis pathogens (Tizard 2000). Neutrophils have bactericidal effects that are mediated through a respiratory burst that produces hydroxyl and oxygen radicals (Heyneman *et al*. 1990). In addition, neutrophils are a source of small antibacterial peptides, defensins, which can kill a variety of mastitis-causing pathogens (Selsted *et al*. 1993). Neutrophils travel from blood to the mammary gland in response to a variety of inflammatory mediators, such as cytokines, complement and prostaglandins (Janeway *et al*. 2001). In this investigation blood neutrophils having more phagocytic ability than milk neutrophils, which substantiates the already existing information of Dang *et al*. (2010) in buffaloes. This may be due to abilities of milk neutrophils to produce ROS, when compared to that of blood (Dosogne *et al*. 2001). The phagocyting ability of the *in vitro* analysis of neutrophil function provides a very effective tool for the study of natural mastitis resistance (MacDonald *et al*. 1994). In this study both blood and milk neutrophil function was diminished during mastitis. A few studies regarding the estimation of phagocytic function of neutrophils from non mastitic bovine milk with low cell counts indicate that alterations in neutrophils function may occur in inflamed udders (Niemialtowski *et al*. 1988). The main reason for the scarcity of studies of neutrophils from non mastitic milk: has been the low numbers of neutrophils in such milk, because most of the existing phagocytic methods require large amounts of cells.

Blastogenic response of blood and milk lymphocytes in normal and mastitic cows

The blastogenic activity of blood lymphocytes is significantly (P<0.001) higher than milk lymphocytes in both normal and sub-clinical mastitic condition (Fig. 3). Con- A induced blood and milk lymphocyte blastogenic response was significantly (P<0.05) lower in sub-clinical mastitic cows than normal animals. This indicated lower activity of lymphocytes during sub-clinical mastitic condition.

Lymphocyte stimulation is widely used to measure immune competence by stimulation of lymphocytes with phytomitogens (Weigel *et al*. 1992). Mitogens used for stimulating T lymphocytes proliferation *in vitro* is Con- A (Kehrli *et al*. 1991) in cattle. In this investigation, both blood and milk lymphocytic activities were lower during subclinical mastitis, which is in accordance with the previous investigations of Owen *et al*. (2000) in cattle. This was realized from the fact that the activity of blood and milk lymphocytes remains depressed during mastitis indicating immune suppression in general as well as in mammary gland.

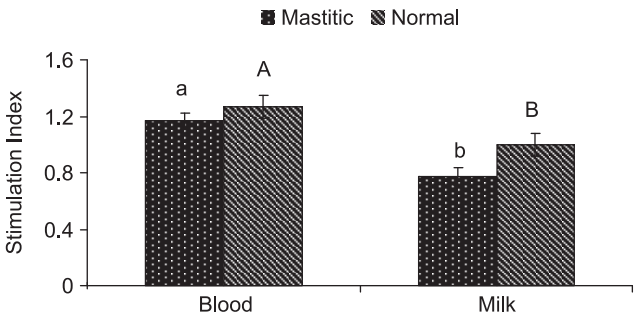

Fig. 2. *In vitro* Phagocytic index of blood and milk neutrophils in normal and mastitic crossbred cows; values are expressed as mean±SEM and the values which lacks a common letter are significantly (P<0.01) different.

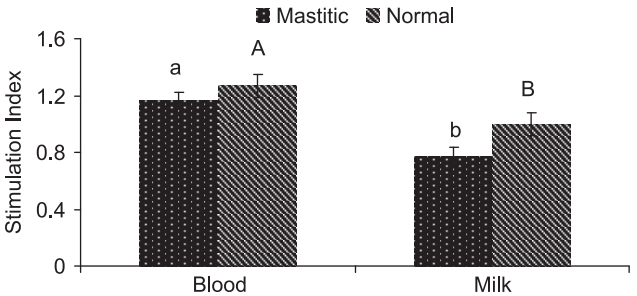

Fig. 3. Concanavalin A induced blood and milk lymphocyte blastogenic response in normal and mastitic crossbred cows; values are expressed as mean±SEM and the values which lacks a common letter aresignificantly (P<0.01) different.

Serum SGPT, SGOT and ALP in normal and mastitic cows

The alteration in serum protein content was nonsignificant (Table 1) but, albumin, globulin ration decreased significantly (P<0.01) during subclinical mastitis. Serum AST level in sub-clinical mastitic cows was significantly (P<0.05) higher than normal cows. There was no significant variation observed in serum AST level in normal and diseased state in crossbred cows. However, in sub-iclinical mastitic cows, the serum ALP was significantly (P<0.01) higher than normal cows

Subclinical mastitis causes considerable changes in milk composition and serum, which may contribute to the impaired immune defense (Megalia *et al*. 2001). Subclinical mastitis increases capillary permeability, which facilitates passage of proteins from blood to milk (Andrei *et al*. 2009). Subclinical mastitis milk shows evidence of direct passage of blood into the milk as indicated by the changes of some blood proteins and enzymes level (Ibtisam El Zubeir 2005). Increased proteins and globulin in the blood of cows indicated an activation of immune response following infection of the mammary gland. These proteins are mainly serum albumin and immunoglobulins that are implicated in udder defense mechanisms (Tsenkova *et al*. 2001). Serum total protein level in normal and mastitic cows in our study is lower than previous reports of Matei *et al*. (2010). In the present investigation albumin, globulin ratio in normal cows was similar as reported by Roussel *et al*. (1972) which significantly (P<0.01) decreased in subclinical mastitis. This finding is in agreement with observations of Benjamin (1978). He reported that globulin fraction increases in bacterial infection. Pandey (2005) also reported correlation between total serum protein (globulins and albumin) and somatic cells count in milk. In the present investigation the level of serum enzymes is in accordance with the report of Matei *et al*. (2010). Only serum SGOT and serum ALP showed significant increase in mastitis. Zaki *et al*. (2008) reported similar findings in buffaloes affected with subclinical mastitis. There are many experimental studies that indicate an increase of serum alkaline phosphatase from mastitis cows, which may suggest that this enzyme plays a role in the pathogenesis of the disease (Vangroenweghe 2004).

The findings from this study permitted us to conclude that the host defense mechanism as well as the udder immunity in terms of *in vitro* neutrophil and lymphocyte activity were compromised during the subclinical mastitis, which was substantiated by the increased level of serum enzyme (AST and ALP) and decreased albumin globulin ratio. Therefore *in vitro* assessment of both blood and milk neutorphil and lymphocyte activity together with the evaluation of SGOT and ALP may be used to detect subclinical mastitis together with other routine diagnostic tools.

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