FULL PAPER *Clinical Pathology*

Transport Stress Increases Somatic Cell Counts in Milk, and Enhances the Migration Capacity of Peripheral Blood Neutrophils of Dairy Cows

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(Received 9 May 2003/Accepted 10 December 2003)

ABSTRACT. The present study was designed to determine the effects of physiological stress on milk-somatic cell counts (SCC) and function of bovine peripheral blood leukocytes (PBL). Nine healthy lactating cows were used in the examination. Five cows were transported 100 km for 4 hr (transported group; TG), and 4 cows were penned (non-transported group; NTG). Blood and milk samples were collected at 0, 2, and 4 hr after loading, and at 2 hr, and 1, 2, 3, and 6 days after unloading. The following activities were mea sured: adhesion receptor (CD 18 and L-selectin) expression of neutrophils and monocytes, migration capacity and percentage of apoptotic cells of neutrophils, serum soluble L-selectin (sL-selectin), plasma cortisol, and SCC. A significant increase in plasma cortisol and milk SCC was observed in TG. Leukocytosis, derived from neutrophils was recorded in TG, and was indicated by apoptotic measurement as an increase of young cells from the marginal pool. Increased migration and decreased surface expression of both L-selectin and CD 18 in neutrophils were observed after transportation. Elevated serum sL-selectin was also noted as a result of transportation. The present study indicated that transport stress modulates peripheral blood neutrophil function, particularly enhancing migration capacity, and c auses diapedesis across the mammary epithelium. Increased milk SCC in transported cattle might be due to these phenomena, and severe physiological stress may bring about an increase in SCC in milk.

KEY WORDS: migration, peripheral blood neutrophil, SCC, stress, transportation.

J. Vet. Med. Sci. 66(4): 381–387, 2004

In a normal non-infected and non-inflamed quarter, the somatic cell population in milk consists of neutrophils, macrophages, and lymphocytes derived from blood circulation, as well as mammary epithelial cells [7]. Inflammatory stimulation caused by invading microorganisms (e.g., *Streptococcus sp., Staphylococcus sp., Escherichia coli*, etc.) introduces pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha; such stimulation also induces several chemokines such as IL-8 to stimulate migration of the peripheral blood leukocytes (PBL) through the milk-blood barrier [35]. Since the number of cells in milk is closely associated with inflammation and udder health, SCC are accepted as the international standard for assessment of milk quality. Such cell counts may precipitate important economic consequences when the price of milk is set according to SCC. However, SCC are known to be increased not only in cases of mastitis, but are also increased by several additional factors, including advancing age and period of lactation [31]. Physical stress is also thought to be an aggregative factor for increasing the SCC [22]. Determination of the precise mechanism involved in these un-inflamed cases of SCC increase is of great importance for the dairy industry.

Transportation is considered to be most stressful to cattle [47]. Previous studies on effect of transport on cattle immunity have primarily measured total and differential leukocyte counts, neutrophil function, and cellular immune function [3, 23, 30]. These studies have shown that transport can result in neutrophilia, along with a modulation of its function. However, definite effects on PBL migration and SCC have not been fully investigated.

The present study was thus designed to analyze the effects of physiological stress on SCC, and to investigate the mechanism of this phenomenon. In the present study we investigated the relationship between milk SCC and PBL function in cows under stress condition loaded 4 hr road transport.

MATERIALS AND METHODS

Animals and sample collection: Nine Holstein-Friesian cows 5 to 8 weeks post-partum, in their second to fifth lactation housed in our station were used. None of the cows had any history of mastitis or peripartum disease. Animals had free access to hay and water, and were fed 8 kg of concentrate daily. These cows were machine milked at 9 am and 4 pm and average milk yield was 20 kg daily at the start of the experiment. All cows were free of udder infections at the time of sampling, and their SCC were normal for respective stage of lactation. These cows were divided into two groups, a transported group (TG; n=5) and a non-transported group (NTG; n=4). One or 2 TG cows were loaded on a 2-ton uncovered truck and transported for 4 hr at an average speed of 25 km/hr around Hitsujigaoka area. Blood samples were collected from the jugular vein at 0, 2, and 4 hr after loading, at 2 hr and 1, 2, 3, and 6 days after unloading. Blood samples for the routine blood examinations were collected in evacuated tubes containing EDTA as an antico-

agulant while and samples for PBL function were drawn in disposal syringe containing heparin as an anticoagulant. Milk samples were also taken at the same sampling intervals as those used for the blood samples. Blood and milk samples from NTG were also colleted at the same times as those used for the TG cows. The blood samples for PBL function were transferred on ice at our laboratory immediately for further analysis. All the procedures were approved by the Laboratory Animal Control Guidelines of the National Institute of Animal Health.

Blood and milk samples: Blood samples for the routine blood examinations were used for the measurement of leukocyte counts and differential counts. Leukocyte counts were determined by the use of an automatic cell counter (Sysmex K-1000; Kobe, Japan). Smears were stained by the May-Grünwald-Giemsa method. Differential counts were determined by counting 100 cells under a microscope. Purified neutrophil suspensions from heparinized-jugular blood were collected by Ficoll-Conray (specific gravity, 1.081) density gradient centrifugation followed by hypotonic lysis of red blood cells and several washes by PBS, as described previously [15]. Based on the May-Grünwald-Giemsa staining and microscopic analysis, cells were routinely determined to be > 95% pure and the viability was determined to be > 98%, as based on Trypan blue exclusion method. The SCC in each milk sample was determined by a Fossomatic counter (Fossomatic 90; Foss Electric, Hiller, Denmark).

Apoptosis assays of neutrophils: Neutrophils from the jugular vein blood were subjected to the terminal deoxynucleotidyl, transferase-mediated dUTP nick-end labeling (TUNEL) technique. The TUNEL technique was performed with a commercially available kit (*In site* Apoptosis Detection kit; Takara, Ohtsu, Japan) used according to the manufacturer's guidelines. The fluorescein isothiocyanate (FITC) labeled neutrophils were resuspended in 1 m*l* of sheath solution and were subjected to analysis by flow cytometer.

Neutrophil migration: Transwell chambers with polycarbonate membranes (3.0 μm pore size, Chemotaxicell; Kurabo, Ohsaka, Japan) attached to a 24 multiwell plate were used for the analysis of neutrophil migration. 1×10^6 cells/m*l* of neutrophils suspended in 400 μl of RPMI-1640 containing 3% FCS were added to the top chamber and these cells were incubated for 2 hr at 37°C and 5% CO_2 under the presence of bovine recombinant IL-8 (100 *n*g/well). Migrated neutrophils were collected from the bottom chamber and washed with PBS. All migrated neutrophils were counted using automatic cell counter and values were expressed as a percentage of 0 hr.

Adhesion receptors on PBL: PBL from the jugular vein were evaluated for adhesion receptor expression. Each 100 μl of whole blood was diluted with 900 μl of ice cold PBS and incubated with 3 μl of mouse antibodies against human CD18 (MHM23; DAKO, Glostrup, Denmark) which recognizes bovine CD18 [34] or 10 μl of bovine L-selectin (MCA1649; COSMO BIO, Tokyo, Japan) for 30 min at 4°C. Cells were washed 3 times with cold PBS, and incubated with FITC-labeled goat $F(ab')^2$ anti-mouse Ig (Southern Biotechnology Associated Inc., Birmingham, AL, U.S.A.) for 30 min at 4°C. After being washed 3 times again with PBS, RBCs were lysed and removed by the hypotonic lysing method and the PBL were resuspended in 1% paraformaldehyde containing sheath solution and were then analyzed by flow cytometry.

Flow cytometry: Fluorescence was measured with an Epics XL (Beckman-Coulter, Hialeah, FL, U.S.A.). The excitation wavelength was 488 nm, and emitted fluorescence was measured between 530 and 560 nm. Dot plots were gated for neutrophils and monocytes by size and granularity. A total of 10,000 events were collected for each sample. TUNEL-positive cells were expressed as a percentage of FITC-positive cells. Surface expression of adhesion molecules was expressed as mean fluorescence intensity (MFI).

Semi-quantitative measurement of serum soluble L-selectin (sL-selectin): Serum sL-selectin was estimated semi-quantitatively by measuring the intensity of anti-bovine L-selectin reactive profiles from the Western blot analysis, *i.e.*, twenty-five μl of each serum sample was diluted with the same volume of PBS, and an equal volume of 2-mercaptoethanol containing sample buffer. The samples were boiled for 3 min, and then the supernatants were collected after centrifugation at 10,000 g for 20 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed by the standard methods. Transferred nitrocellulose membranes were reacted with × 250 diluted anti-bovine L-selectin, and subsequently reacted with × 50,000 diluted Horse radish peroxidase (HRP)-labeled anti-mouse Ig (ZYMED, South San Francisco, CA, U.S.A.). HRP probes were detected by a chemiluminescent detection system (Amersham, Buckinghamshire, U.K.). Image capture and analysis were performed using Electrophoresis Documentation and Analysis System 120 (Kodak Digital Science, NY, U.S.A.), and the intensity of sL-selectin was expressed as a percentage of 0 hr.

Cortisol measurements: Plasma cortisol concentrations were determined by automatic magnetic particle enzyme immunoassay (Kainos Lab. Inc., Tokyo, Japan).

Statistical analysis: Comparison of neutrophils, SCC, apoptotic cells and migration of neutrophils, and surface expressions of adherence molecules of either neutrophils or monocytes between the TG and NTG, were carried out using the mixed-models procedure for measures repeated across time [14]. SCC data were transformed to $\log_{10}$. Data were analyzed using the general linear models procedure for SAS and were tested by least squares ANOVA (SAS/STAT Software Release 6.11; SAS Inst., Inc., Cary, NC, U.S.A.).

RESULTS

Cortisol: Changes in plasma cortisol relative to transportation are shown in Fig. 1. Significant increase in plasma cortisol levels were observed during transportation

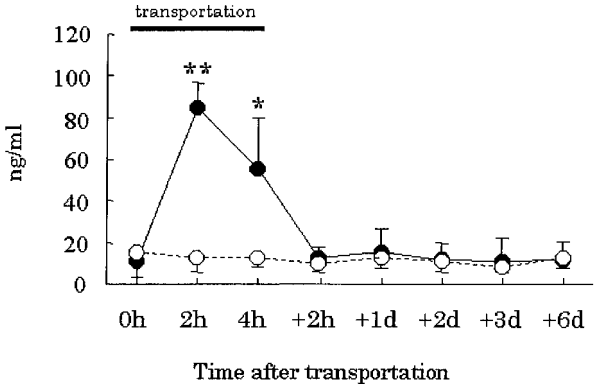


Fig. 1. Changes in plasma cortisol concentration in transported (n=5; ●) and non-transported (n=4; ○) cows. At a given time point, * or **indicates a significant difference from that of non-transported cows (* $P<0.05$, ** $P<0.01$). Bars indicate standard error. Where not visible, bars fall within data points.

($P<0.001$) and maximum value was recorded at 2 hr (0 hr vs. 2 hr = 11.4 ± 3.9 vs. 84.9 ± 12.1 ng/m*l*). There were no definite changes in NTG cows.

Response and changes in the function of blood PBL after transportation: A slight decrease (2–5 kg) in milk production in the TG cows until day 1 after unloading was observed. No clinical findings including anorexia, fever, and weariness, were observed during and after transportation. A significant leukocytosis was observed in TG until 2 hr after unloading. Based on differential leukocyte counts, the increase was due to an elevation in neutrophils and was 2.1-time more than the value ($P=0.005$) of NTG at 2 hr after unloading (Fig. 2). Neutrophils showed normal segmented forms with no left shift. Before the examination, the percentage of apoptotic cells in the jugular vein-derived neutrophils were 11.8 ± 3.3% (TG) and 17.6 ± 1.6% (NTG). This value of TG decreased constantly until day 1 after unloading ($P<0.001$), and showed a minimum value (1.2 ± 0.3%) at 2 hr after unloading (Fig. 2). However, no definite changes in the number of PBLs and apoptotic neutrophils were observed in NTG. The expression of L-selectin in neutrophils (Fig. 3) decreased constantly and a minimum value (41% MFI of that before transportation) was observed on day 1 after unloading ($P=0.04$). A slight decrease of CD18 expression in neutrophils was observed at 4 hr ($P<0.05$) in TG (Fig. 3). There were no definite changes in expression of both L-selectin and CD18 molecules in monocytes. An increase in neutrophil migration was observed during transportation ($P=0.03$), and 2–3 days ($P<0.05$) after unloading, although the number of migrated cells varied individually (Fig. 4).

sL-selectin: Changes in serum sL-selectin in accordance with transportation are shown in Fig. 4. sL-selectin of TG clearly increased at 2 hr ($P=0.02$) and 4 hr ($P<0.001$), and showed a tendency to increase until 2 days after unloading. During these periods, sL-selectin showed a 150–200% increase in values than those observed before transportation.

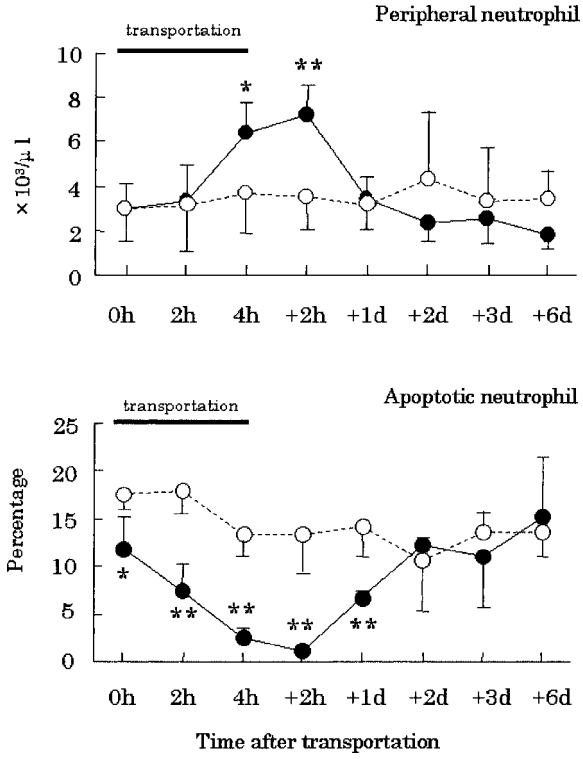


Fig. 2. Changes in number of peripheral neutrophils and percentage of apoptotic neutrophils in transported (n=5; ●) and non-transported (n=4; ○) cows. At a given time point, * or **indicate significant difference from that of non-transported cows (* $P<0.05$, ** $P<0.01$). Bars indicate standard error.

There were no changes in NTG values.

SCC: Changes in SCC in relation to transportation are shown in Fig. 5. A significant increase in SCC was observed during transportation ($P<0.001$), and the maximum values were recorded at the end of transportation (0 hr vs. 4 hr = 20 ± 15.2 vs. 338 ± 121 × 10^3 cells/m*l*). Increased SCC was also observed in NTG; though, the increase was significantly lower than the TG cows.

DISCUSSION

Stress responses have generally been demonstrated to increase the production of ACTH and beta-endorphins via the pituitary, which is under the control of the following hypothalamic hormones: corticotrophin releasing hormone (CRH), oxytocin, and vasopressin; resulting in an increased secretion of glucocorticoids by the adrenal cortex [19]. It is also-known that transportation is the most frequent stressor in cattle management that induces a rise in plasma cortisol and also change in leukocyte profiles such as leukocytosis with neutrophillia, lymphopenia, eosinopenia, and changes in leukocyte function during and/or after transportation [24, 30, 39]. In our experiment, the results concerning leukocyte profiles and cortisol levels in TG were in agreement with the above observations. Similar phenomena were observed by

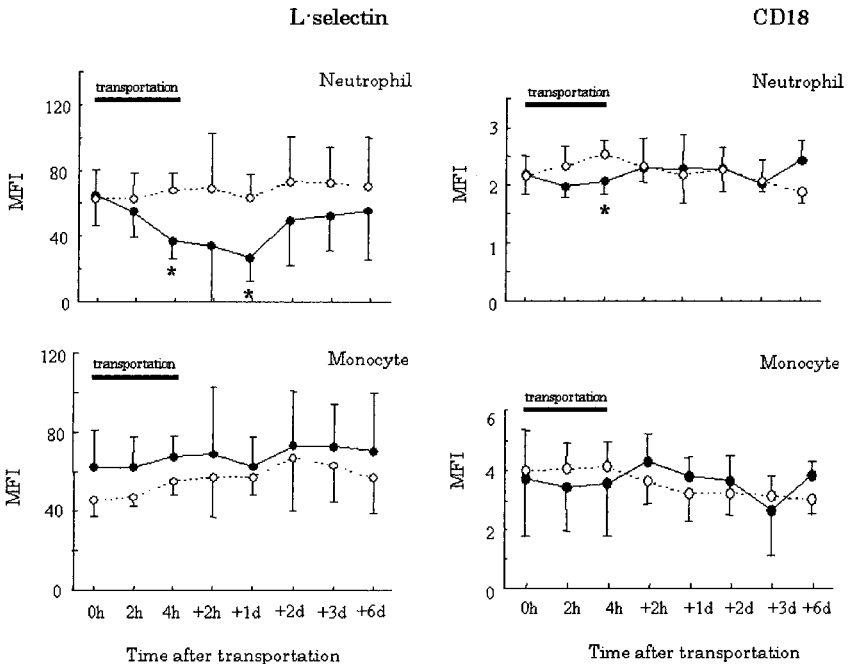
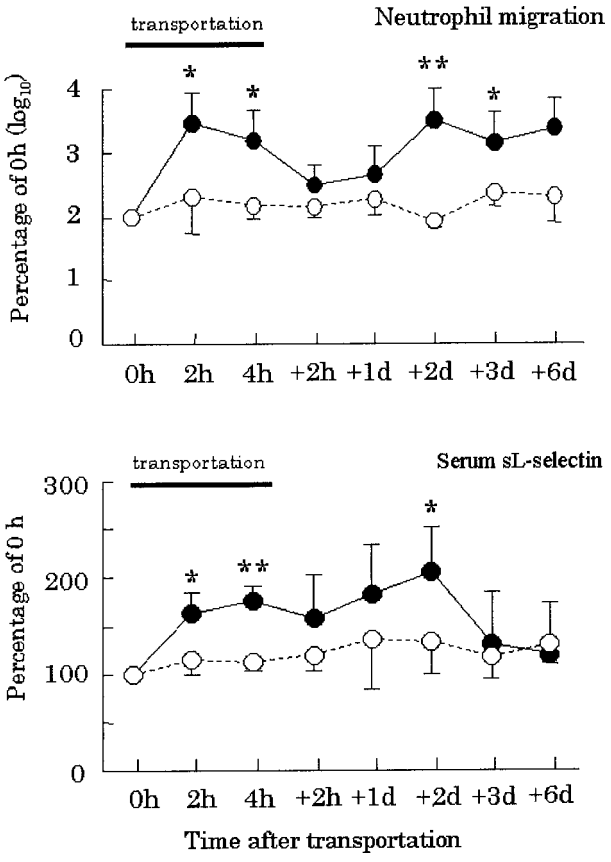


Fig. 3. Changes in surface expression of L-selectin and CD18 on PBL in transported (n=5; ●) and non-transported (n=4; ○) cows. At a given time point, *indicate significant difference from that of non-transported cows (* $P<0.05$). Bars indicate standard error.



administration of ACTH and corticosteroids, and have been reported to be due to the input of neutrophils from the marginal pool [38]. The properties of increased neutrophils are not clear. However, the actual number of band neutrophils has been shown to be low, which does not qualify as a left shift; such cases are thought to involve mature cells [6, 38]. In this experiment, we found a transient but significant decrease in the number of apoptotic neutrophils (11.8 ± 3.3%; 0 hr vs. 1.2 ± 0.3%; 4 hr) with transportation, as shown by the TUNEL flow cytometry method; these results were inversely related to neutrophillia. The TUNEL flow cytometry method is a highly sensitive method of detecting apoptotic cells, and spontaneous apoptotic neutrophils can be detected with this method at percentage of less than 10 percent of circulating neutrophils [18]. This value was comparable with results at 0 hr obtained from the cows in the present examination. These spontaneous apoptotic neutrophils are thought to be aged neutrophils [13]. Neutrophils appearing in the circulation due to transportation might be young cells from the marginal pool, and the relative percent-

Fig. 4. Changes in neutrophil migration and serum soluble L-selectin (sL-selectin) in transported (n=5; ●) and non-transported (n=4; ○) cows. Serum sL-selectin was measured semi-quantitatively by the intensity of Western blotting profiles. Data were expressed as a percentage of the value obtained before the examination (0 hr). At any given time point, * or **indicate a significant difference from that of non-transported cows (* $P<0.05$, ** $P<0.01$). Bars indicate standard error. Where not visible, bars fall within data points.

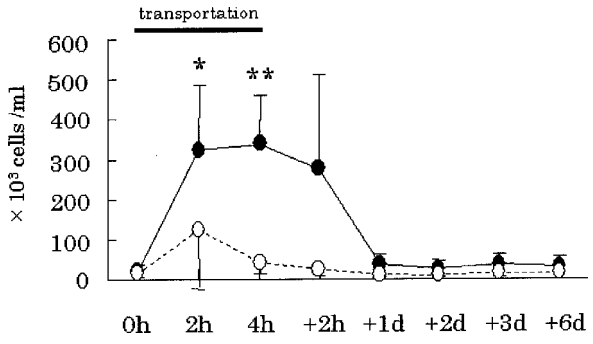


Fig. 5. Changes in milk somatic cell counts in transported (n=5; ●) and non-transported (n=4; ○) cows. At a given time point, * or **indicates a significant difference from that of non-transported cows (* $P<0.05$, ** $P<0.01$). Bars indicate standard error. Where not visible, bars fall within data points.

age of aged neutrophils decreased with an increase in the number of these neutrophils in the circulation.

One of the major findings of the present experiment was that milk SCC were increased by transport stress. Contradictory results regarding the relationship between milk SCC and stress, or stress-mediated hormones, have been reported. Several groups have failed to detect increases in milk SCC from mastitis-free cows following either ACTH or adrenal corticoid injection [8, 32, 33]. However, some authors have demonstrated stress-induced increases in milk SCC [44, 46]. Furthermore, Wegner *et al*. [45] found increased milk SCC in both corticotropin-injected and heat-stressed cows, both with no evidence of current mastitis. A similar increase in bulk-milk SCC was observed in cows stressed by intergroup movement [22]. The effects of stress-mediated hormones on SCC were equivocal, though several physical stressors are thought to increase milk SCC. The major components of milk SCC are blood-derived cells, neutrophils, macrophages, and lymphocytes [7]. Therefore, increased SCC can be explained by enhanced migration of PBL from an intravascular site to mammary duct vessels, or by PBL movement from blood into milk via leaky tight junctions. Stress may reduce mammary tight junction leakiness [27, 42], and we were unable to detect increased milk BSA in this experiment (data not shown). BSA is not produced in the mammary gland, and its presence in milk can only be explained by movement from blood to milk via leaky tight junctions [41]. Therefore, the latter possibility is probably negligible.

Both L-selectin and CD18 are known to play important roles in leukocyte migration from the blood to sites of inflammation. L-selectin mediates leukocyte rolling on the endothelium and possesses a lectin domain that interacts with carbohydrate moieties on glycoproteins and glycolipids of the vascular endothelium [21]. A unique feature of L-selectin is that it is shed from the cell surface following leukocyte activation by inflammatory stimuli (*e.g.*, lipopolysaccharide, IL-1, IL-8, and platelet-activating factor) [2, 9, 17, 26] and it is present in the circulation as sL-selectin. Therefore, PBL-enhanced transmigration through the endothelium to sites of inflammation lead to increased plasma sL-selectin *in vivo* [20, 25]. CD18 is a β subunit of Mac-1 which is a member of β_2-integrin family and noncovalently linked to α subunit (CD11b). Hyperadherence of the neutrophil is CD-18 dependent and is mediated by Mac-1 binding tightly to its ligands, intercellular adhesion molecule 1, on the vascular endothelium. There is little known about affection of physiological stress on bovine adhesion molecules. Decreased L-selectin and CD18 in neutrophil are observed in the case of administration of dexamethasone [4, 5]. Decreased surface expression of L-selectin in circulating neutrophil is also reported in bovine mastitis [11, 28, 36]. The reliable reason of these phenomena is not clear, however some scientists speculate that dexamethasone and/or glucocorticoid produced by acute inflammatory stimuli mediate these changes. Conformational changes in these adhesion molecules [5], activation of the proteolytic enzyme system involved in L-selectin cleavage [4], or the appearance of young cells from marginal pool in the circulation [5, 40] are thought to sequential occur following these stress hormone production.

In the present study, enhanced neutrophil migration was observed during or after transportation, which was accompanied by decreased expression of L-selectin on neutrophils, consequently leading to increased serum sL-selectin levels. In this examination, we only provided semi-quantitive data of sL-selectin using Western blot analysis. However, in our preliminary examination, we measured sL-selectin in the supernatant of serial diluted PBL cells, which were stimulated with PMA or IL-8, and the levels were fairly related to the no. of cells incubated (data not shown). Therefore, obtained sL-selectin data in this examination should sustain the further analysis. The findings obtained here, indicate the transmigration of neutrophil from an intravascular site to an extravascular site, stimulated by transport stress without inflammatory stimulus. Contradictory results have been reported regarding the effects of glucocorticoids and dexamethasone on leukocyte migration. Some groups have reported that these hormones enhance migration capacity [37, 38] and chemotaxis [1]; while other report [12] do not show such results. Therefore, it remains difficult to attribute increasing transmigration of neutrophil during transportation to an increased level of cortisol. It is gradually becoming evident that stress does not simply suppress all aspects of immunity by activation of the HPA axis, but rather results in a variety of changes in acute and chronic immunocompetence, and even in exaggerated responsiveness of certain components of the immune/inflammatory reaction. Regarding these aspects of study, activation of the sympathetic nervous system by several stressors produced peripheral immune CRH [12], lymphocytic ACTH [10], epinephrine, and norephinphrine modulate immune regulating cells, including circulated leukocytes. Neutrophil-priming cytokines (IL-6, IL-8, and G-CSF) also increased, and induced neutrophil mobilization upon endurance exercise in humans [43]. An increase in the number of neutrophils in broncho-

alveolar fluids was observed in calves stressed by forced walking and subsequent dipping in cold water [16]. Such evidence indicates that some transmigrating factors is released under conditions of stress, leading to enhanced migration of neutrophil from the circulation into extravascular fluid spaces, including mammary duct vessels and alveolar cavities. Increased SCC during transportation might be a part of the expression of these enhanced PBL transmigrations.

These finding indicate that severe physiological stress may bring about an increased SCC in milk. However, further studies are required to confirm this finding.

ACKNOWLEDGMENT. This work was supported by a grant for a research project entitled "Salmonella Control on Farms" coordinated by the Ministry of Agriculture, Forestry, and Fisheries, Japan.

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