Change in Lymphocyte Subsets of Holstein Calves Caused by Short-term Transportation

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Abstract

In order to study the possibility of using lymphocyte subsets as a stress indicator, the changes of the subset proportion in peripheral blood were examined after transportation. Six 3-month-old male Holstein calves were transported for about 3 h in a lorry via an ordinary road. Blood samples were collected at the expected unloading time on the day before transport (T0), immediately after unloading (T1), 24 h after unloading (T2), and 1 week after unloading (T3). CD2, CD4, CD8, CD21, CD25, WC1, and NKp46 antigens on peripheral blood mononuclear cells were measured by flow cytometry. There was an increase in the proportions of CD25+ cells and WC1+ $\gamma\delta$T cells at T1 and T2. The proportions of NK cells increased at T1, including an increase in the population of CD2- NK cells. CD2+ NK cells did not show any change. The proportions at T1 were lower than at T3 in CD21+ B cells. CD4+ and CD8+ $\alpha\beta$T cells did not change significantly, although CD2+ T cells showed a trend toward reduction at T1. Consequently, CD2- NK cells and WC1+ $\gamma\delta$T cells might offer potential as stress index for calf transport.

Discipline: Animal industry
Additional key words: $\gamma\delta$T cell, NK cell, transport stress, cattle

Introduction

The concept of animal welfare for farm animals in Japan remains in the initial stages, but is slowly becoming popular among consumers. Although a practice guideline to be followed by farmers exists (Japan Live Stock Technology Association 2011), there is further need for scientific methods of assessing the condition of animals. Cattle experience various kinds of stress, but transport is considered one of the most stressful events. Transport is known to affect the immune system of cattle via many factors (Swanson & Morrow-Tesch 2001, Knowles & Warriss 2007). There are various immunological or hormonal indices for a study on stress, although a perfect index does not exist. For example, the level of cortisol in the blood has been recognized as a representative indicator of stress, including the elevation caused by acute stress as well as the low level resulting from chronic stress (Christison & Johnson 1972). However, the acute stress-related increase often masks other stressors and confuses the effect, since the increase begins within a few minutes and rapidly reaches a peak. Therefore, novel indices have always been required for a study.

Lymphocyte subpopulation analysis is a newer immunological index for stress in cattle (Van Kampen & Mallard 1997, Anderson et al. 1999, Fell et al. 1999). This analysis may possibly express the effect from chronic environmental stress in addition to that from acute stress. Nevertheless, only a few studies have examined transport stress (Riondato et al. 2008, Ishizaki & Kariya 2010). Long-distance transport has increased in Japan, although short distance transport is more common in many areas. Young cattle have traditionally been entrusted to public farms in each area, so

as to reduce the feed cost and each farm household's work. Recently, some prefectures have also begun to recommend intensive grazing for the effective utilization of abandoned farmland. The aim of this study was the verification and development of a study on lymphocyte subsets for few hours of transport.

Materials and Methods

This study was carried out in strict accordance with the guidelines approved by the Animal Care and Use Committee of the National Institute of Livestock and Grassland Science (Permit Number: 10032415). All efforts were made to minimize suffering.

Six 3-month-old male Holstein calves, each weighing approximately 100 kg, had been raised in group feeding. Standard formula feed and hay had been given to the calves, because their rumen develops to the same level as that of adult cattle at three months after birth. The calves were individually tied in a covered 8-ton lorry ($>6.2 \times 2.1$ m) without bedding, and transported via an ordinary road. The journey from the farm (in Maebashi) to the National Institute of Livestock and Grassland Science (in Tsukuba) took about 3 h (110 km). While traveling, the calves were able to lie or stand. On the day before transport, blood samples were taken by external jugular venepuncture at the same time as the expected arrival time on the next day (10:00; T0). Upon arrival, samples were collected immediately after unloading (10:00; T1), 24 h after unloading (T2), and 1 week after unloading (T3). The animals developed no clinical abnormalities during the experiment. After unloading, the calves were housed in individual indoor stalls that enable direct contact between the animals.

Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep (Axis-Shield Poc AS). The labeled antibodies were commercialized mouse monoclonal antibodies conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC), or Alexa Fluor 647 (AbD Serotec). Table 1 shows the specificities and clones of the antibodies used for the flowcytometric phenotyping of lymphocyte subpopulations. About 100 µL of 1.0×10^6 separated PBMC from each sample were incubated for 20 min with monoclonal antibodies at 4°C in the dark. Cells were then washed and resuspended in 1 mL of PBS. A minimum of 50000 events from a gated lymphocyte population were acquired and analyzed using a Gallios Flowcytometer (Beckman Coulter Inc.). FlowJo software (Tree Star Inc.) was used for data analyses based on the cell surface marker expression.

Plasma was separated using a refrigerated centrifugation ($2200 \times$ g for 30 min at 4°C) and then stored at -30°C. Cortisol concentration was measured using a commercial EIA kit with a QUARTUS ImmunoCube Auto-Analyser (Kainos Inc.).

Analysis of variance was performed using a repeated-measures mixed linear model (MIXED procedure) of SAS Add-In 6.1 for Microsoft Office (SAS Institute Japan Ltd.) in order to compare temporal variation. Day was included as a fixed effect and individual calves as a random effect in the model. Multiple comparisons for each effect were controlled for using the Tukey method and *P < 0.05* was considered significant.

Results

The counts of PBMC were constant ($2\text{-}5 \times 10^6$ /ml whole blood) and did not change significantly in any of the animals. Alterations in the percentage of lymphocyte subsets expressing the selected differentiation antigens are shown in Fig. 1. The proportion of CD2+ cells in PBMC was the highest at all points in this study (Fig. 1A). It showed a trend toward reduction at T1 (*P = 0.06*), as compared with at T0. The proportions of CD21+ B cells increased at T3, as compared with at T1. CD4+ and CD8+ αβT cells, did not change significantly.

The proportion of CD25+ cells was increased at T1 and remained significantly high at T2, as was the case for WC1+ γδT cells (Fig. 1 B). In contrast to CD4+ or CD8+ αβT cells (including the αβ chain in the receptor), γδT cells have another type of receptor consisting of the γδ chain. And most bovine γδT cells have a surface molecule stainable by the WC1 antibody (Pollock & Welsh 2002). The proportion of WC1+ γδT cells increased significantly at T1, and at T3 returned to the same level as at T0. There was a significant increase in the total proportion of NK cells between T0 and T1. The proportion of CD2- NK cells also increased significantly at T1 (Table 2). CD2- NK cells at T3 were lower than that at T1. The proportion of CD2+ NK cells did not show any change.

Plasma cortisol concentration showed a significant increase at T1, and returned to the T0 level at T2 (Table 3). The value at T3 was significantly lower than that at T0.

Discussion

The transport of live cattle is known to be stressful, and can lead to immune suppression, particularly in calves (Swanson & Morrow-Tesch 2001). Meanwhile, it is not yet clear whether changes in the relative proportions of lymphocyte subsets in cattle follow a circadian rhythm, as cortisol does (Riondato et al. 2008). Therefore, prior to transport, the first sample was collected at the same time of day as the expected arrival time after transport on the following day.

It has been reported that short-term exposure to acute psychological stress results in an increase in γδT cells in

Table 1. Explanation of antibodies

Specificity	Clone	Fluor	Cellular expression
CD2	CC42	FITC	αβT cell, γδT cell*, NK cell
CD4	CC8	FITC	αβT cell
CD8	CC63	Alexa Fluor 647	αβT cell
CD21	CC21	PE	B cell
CD25	ILA111	PE	B, T, NK cell
WC1	CC15	FITC	γδT cell
NKp46	AKS1	PE	NK cell

The specificities and clones of antibodies used for the flowcytometric phenotyping of lymphocyte subpopulations (PE: phycoerythrin, FITC: fluorescein isothiocyanate), and the objective cells. * low ratio.
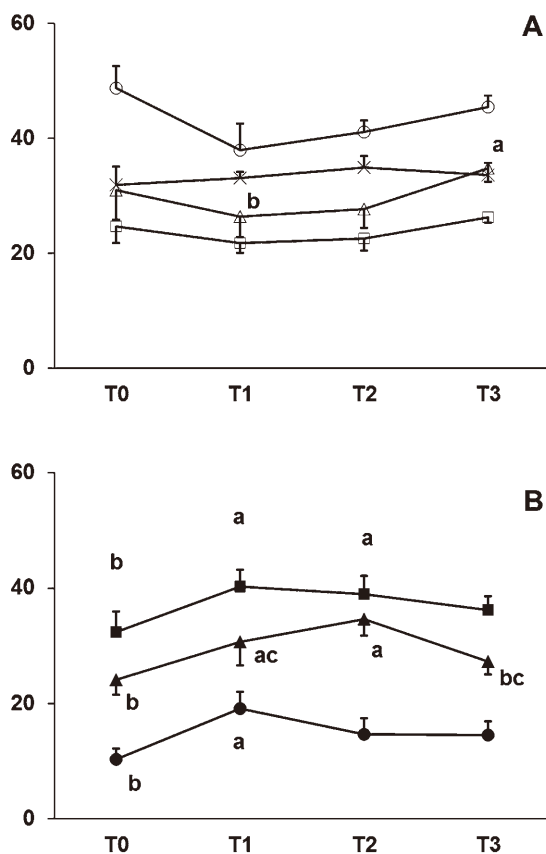
Fig. 1. Proportion of lymphocyte subset in PBMC.
The percentage of circulating lymphocyte subsets in the peripheral blood mononuclear cells (PBMC) of calves before departure (the same time as the expected arrival time on the next day (10:00; T0)), immediately after unloading (T1), 24 h after unloading (T2) and 1 week after unloading (T3). Values are presented as mean ± SE (n = 6). The subsets are shown as cells that are positive for an antibody to A. CD2 (open circle), CD8 (cross), CD21 (open triangle), or CD4 (open square); B. CD25 (closed square), WC1 (closed triangle), or NKp46 (closed circle) antigens. Different subscript letters indicate significant differences in the population (P < 0.05).

humans (Anane et al. 2010), although in calves, a decrease after 14 h of transport was shown by Riondato et al. (2008). The opposite result observed after 3 h of transport in this study may be due to different durations of transport, and the intensity of stress. The significantly high levels of WC1+ γδT cells remained evident the next day. Baldwin et al. (2000) reported a significant fluctuation in the proportion of γδT cells coinciding with an environmental change. In this study, calves were not only transported but also moved to a different farm. γδT cells are the first line of immunity in young animals, similar to NK cells (Pollock & Welsh 2002). It is possible that a novel environment, possibly including exposure to new microbes, affected increases in the WC1+ γδT cell population at T2.

Because NK cells have low percentage of white blood cells (Graham 2009), detecting the clear variation caused by stress was considered difficult. Therefore, we used PBMC, excluding granulocytes occupying the greater part of the white blood cell population. The significant increase in the proportion of NK cells was evident at T1. It has been reported that the number of circulating NK cells peaked shortly after 2 h of transport (Ishizaki & Kariya 2010), suggesting that a few hours of transport can result in an increase in NK cells. The CD2 molecule is the receptor for activation on both T cells and NK cells (McNerney & Kumar 2006), although it is not expressed on most WC1+ γδT cells (Davis et al. 1996). Storset et al. (2004) reported that in cattle, most circulating NK cells are CD2+ and that CD2- cells comprise a low percentage. In this study, only the smaller CD2- NK cell population increased significantly just after transport, as did the total NK cell population. It has been reported that the CD2- subset shows signs of being more activated in circulation (Boysen et al. 2006). Thus, the CD2- subset may react rapidly and more clearly than the CD2+ subset (Riondato et al. 2008). Meanwhile, the proportion of CD2- NK cells in this study showed a decrease between T1 and T3. This may be due to change ahead of the

Table 2. Proportion of NKp46+cells in PBMC

	T0	T1	T2	T3
Total NK	10.3 ± 1.8^b	19.1 ± 2.8^a	14.7 ± 2.8	14.5 ± 2.4
CD2+NK	8.2 ± 1.6	11.9 ± 2.8	9.8 ± 1.9	11.0 ± 2.1
CD2- NK	2.1 ± 0.2^b	7.2 ± 1.0^a	4.9 ± 1.0^{ab}	3.5 ± 0.4^{bc}

The percentage of total NK cells (detected with NKp46 antigen), CD2+NK cells, and CD2-NK cells in the peripheral blood mononuclear cells (PBMC) of calves before departure (T0), immediately after unloading (T1), 24 h after unloading (T2), and 1 week after unloading (T3). Data are presented as the means ± SE (n = 6). Different subscript letters indicate significant differences in the population ($P < 0.05$).

Table 3. Changes in plasma cortisol

T0	T1	T2	T3
2.89 ± 0.58^b	4.39 ± 0.64^a	1.54 ± 0.17^{bc}	0.85 ± 0.32^c

Changes in the concentration (μg/dL) of plasma cortisol (n=6) before departure (T0), immediately after unloading (T1), 24 h after unloading (T2), and 1 week after unloading (T3). Data are presented as the means ± SE (n = 6). Different subscript letters indicate significant differences ($P < 0.05$).

total NK population, affected by a new environment. Then there is also the possibility that only CD2- NK cells are able to respond to such environmental stimuli sensitively, as both γδT and NK cells play important roles in the innate immune system.

CD25, the α-chain of the IL-2 receptor, is expressed on activated T-cells, B-cells, and NK cells (Waldmann et al. 1998). Thus, the increase in CD25+ cells observed in this study may have resulted from an increase in CD25 on WC1+ γδT cells and NK cells.

Long-term (14 h) transport decreased the proportion of CD4+ and CD8+ T cell just after arrival, although the decrease of the CD21+ B cell did not occur until the next day (Riondato et al. 2008). The results of the CD4+ T and CD8+ T cells did not show a significant change after transport in this study. Therefore, the αβT cell may not vary the proportion in a short time. In contrast, the CD21+ B cell at T3 was significantly higher than at T1. Short transport and the succeeding new environment may affect the B cell more clearly than the αβT cell, although the effect to the cell may also vary due to distance.

A positive correlation between circulating NK cell counts and plasma cortisol levels after transport has been reported (Ishizaki & Kariya 2010). Although the relationship and the mechanism of action remain unclear, the reaction to stress in the lymphocyte subpopulation is thought to be slower than the rapid increase in cortisol. The stress may have already begun before loading, because the novelty of

encountering unfamiliar humans and the behavior witnessed by calves for the first time are strong stressors for them (Grandin 2007). The increase in cortisol at T1 observed in the current study suggested that arrival time was when the animals were experiencing severe stress. However, a higher level at T0 than at T2 and T3 suggests that pre-transport stress may already have begun on the previous day. Novel human behavior and the first capture for collecting blood might affect the cortisol concentration at T0 in a sensitive calf, although other indices could not represent the results from an acute stressor until blood was collected. Then the cortisol levels had decreased significantly a week after arrival. Habituation due to our repeated handling and restraint for approximately 1 week might have been the cause of the decrease, functioning to lower the basal cortisol level (Grandin 2007). Furthermore, the decline and low level of cortisol could also have induced the decrease in CD2- NK cells at T3.

In summary, we substantiated that a few hours of transport can lead to an increase in bovine NK cells. In particular, CD2- NK cells might reflect the effect of transport on stress levels. Moreover, as well as NK cells, the study indicated the possibility that γδT cells could also be a useful index for short-term transport. It is still unknown how the lymphocyte population may change depending on stress from the breeding environment. To improve livestock welfare, further detailed studies on the associations between stress and the lymphocyte indices are required.

Acknowledgements

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