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## The effects of stressors on lymphocyte populations and function in lactating dairy cows.

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### ABSTRACT

Increased stress in animals can lower immune system function and hence decrease resistance to disease. The objectives of the study were to determine the effects of two stressors on lymphocyte function in lactating dairy cows. Jersey cows were allocated to four, 7-day treatments in a 2 x 2 factorial plan. The factors were level of feeding (4% bodyweight vs 3% bodyweight) and lying time (free choice lying vs prevention of lying from 1500 to 0645 h daily). Blood samples collected during the pre-treatment and treatment periods were analysed for *in vitro* lymphocyte proliferative response and sub-populations. Lymphocyte proliferative response was not affected by feed level or lying time. Only minor changes occurred in lymphocyte sub-populations. Within the fully-fed cows, lying deprivation resulted in a greater proportion of CD8 (suppressor) cells. These data indicate that lymphocyte function in lactating cows may not be compromised by medium term restrictions in feeding and lying, and that any stress-induced decreases in cell-mediated immunity which occur in practice, are the result of more severe, or prolonged, conditions.

**Keywords:** cattle; lymphocytes; stress; nutrition.

### INTRODUCTION

The mammalian immune system may be suppressed by increased stress levels (Griffin, 1989). Such mechanisms are likely to be important in dairy cows, in which challenges from micro-organisms can cause mastitis and lameness conditions, thus adversely affecting animal welfare, productivity and longevity within the herd.

Lymphocytes are the key white blood cells controlling the immune response. Studies in cattle have shown that externally-acting stressors can reduce lymphocyte blastogenesis and render animals more susceptible to disease (Blecha and Minocha, 1983). Such lymphocyte tests in cattle measure changes in functional and proliferative capacity (Kelley *et al.*, 1981). Extensive studies in humans and rodents have demonstrated that psychological and physical stressors alter both lymphocyte activity and the proportions of lymphocyte sub-classes, particularly the ratio of helper (CD4) and suppressor (CD8) subtypes (Rook *et al.*, 1994). The  $\gamma\delta$  lymphocyte is an important contributor to immunity at body surfaces, and has been shown to be depleted in cattle suffering from thermal stress (Morrow-Tesch *et al.*, 1996). Monoclonal antibodies to all these subtypes are now available for cattle, and the use of flow cytometry allows the rapid and accurate enumeration of these cell types.

Most experiments examining the links between stress and immune system function have studied acutely-acting stressors. In commercial farming practice, stressors are often of a milder, more chronic nature, and are often multiple. Dairy cows may be subject to less than optimal feed availability at certain times of the year. Further, the ability of animals to cope with difficult environments may be reduced when energy requirements are not fully being met through feed intake. Dairy cows in New Zealand have been

noted to be reluctant to lie down when held in wet or muddy conditions and deprivation of lying has been successfully utilised elsewhere for studying stress responses in cows (Munksgaard and Simonsen, 1996).

The aim of this study was to determine, under high and low levels of feeding, the lymphocyte responses of dairy cows to an additional stressor, deprivation of lying. The hypothesis was that lactating cows subjected to five to seven days of reduced lying would have lowered lymphocyte proliferation and CD4:CD8 lymphocyte subtype ratio. We further hypothesised that these effects would be enhanced by a sub-optimal feeding level.

### MATERIALS AND METHODS

#### Animals and treatments

For details, refer to Verkerk *et al.*, (1999).

#### Lymphocyte proliferation

Blood samples were collected on days 7 and 8 of the pre-treatment period and days 5 and 6 of the treatment period, and analysed for *in vitro* lymphocyte proliferative response. On each sampling day, 8 cows (2 from each treatment cell) were sampled. Blood was collected via indwelling jugular catheter into sterile tubes containing lithium heparin as anticoagulant. Under sterile conditions, 15 ml of heparinised blood was added to 15ml of Hanks Balanced Salt Solution (HBSS; Sigma) and then this mixture was overlaid onto 10 ml of Ficoll-Conray (SG = 1.081 ± 0.004). Following centrifugation at 400 x g for 60 minutes, the resulting lymphocyte layer was washed once in HBSS and then re-suspended in RPMI 1640 culture media (Gibco) containing 10% red deer serum.

Lymphocytes were diluted to a final concentration of 2.5 x 10<sup>6</sup> cells per ml and added (100  $\mu$ l per well) to a

96-well culture plate, together with 50 µl of media or media containing 50 µg/ml Concanavalin A. The plates were incubated at 37°C and 5% CO<sub>2</sub> for 48 hours. The cell proliferation label bromodeoxyuridine (BrdU) was added to each well (15 µl of 100 µM BrdU in culture media), before the plates were incubated for a further 24 hours. The plates were centrifuged before the media was removed and then cell proliferation was measured by assaying BrdU incorporation using an ELISA method, with optical density measured at 450nm (Boehringer Mannheim, Germany).

### Lymphocyte sub-populations

Blood samples were collected on day 7 of the pre-treatment period and day 5 of the treatment period, and analysed for lymphocyte sub-populations represented by the cell surface antigens CD2 (universal lymphocyte marker), CD4 (T helper), CD8 (T suppressor), and γd. Whole blood (50 ml) was collected via jugular catheter into a sterile syringe containing acid citrate dextrose as anticoagulant. Mononuclear cells, were obtained by centrifugation on Histopaque 1077 (Sigma). The cells were then prepared for two-colour immunofluorescence assay by incubation with monoclonal antibodies against the cell-surface antigens CD4, CD8, CD2 and γd (VMRD, Pullman), and labelled with isotype-specific, fluorescein or phycoerythrin-labelled second antibody. Labelled cells were counted with a FACScan (Becton-Dickinson Immunocytometry Systems). Data on 5000 cells per sample were analysed.

### Statistical analyses

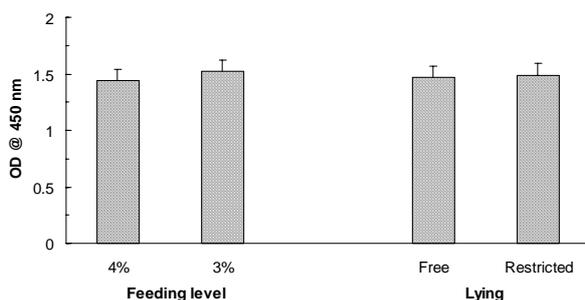
Data were analysed for a 2 x 2 factorial plan with two replicates, using the General Linear Model of SAS. Pre-treatment values were included as covariates in the model. Lymphocyte proliferation data were analysed with data of sampling also included in the model.

## RESULTS

The behavioural observations showed that lying-deprived cows lay down for 4 ± 0.3 hours during a 24-hour period, whereas free-lying animals lay down for 9 ± 0.8 hours.

There were no effects ( $P > 0.05$ ) of feeding level or deprivation of lying on *in vitro* lymphocyte proliferation (Figure 1).

**FIGURE 1:** Effects of feeding level (4 % bodyweight vs 3% bodyweight) and lying (free-choice vs restricted) on lymphocyte proliferation in lactating dairy cows (n = 32) as measured by optical density at 450 nm.



The effects of treatments on lymphocyte sub-populations are presented in Table 1. There was a tendency ( $P < 0.09$ ) for lying-restricted cows to have a lower proportion of lymphocytes (CD2) than unrestricted animals. A tendency for a feeding x lying interaction ( $P < 0.06$ ) was present for the proportion of T suppressor (CD8) cells, and fully-fed lying-restricted cows had a greater ( $P < 0.05$ ) proportion of CD8 cells than fully-fed free lying animals (6.0 vs 4.6%, respectively; pooled sem = 0.46).

**TABLE 1:** Effects of feeding level (4 % bodyweight vs 3% bodyweight) and lying (free-choice vs restricted) on lymphocyte types in lactating dairy cows (n = 32).

Cell type	Feeding			Lying			Significance		
	4%	3%	sem	Free	Restricted	sem	Feeding	Lying	Feeding x Lying
CD2 (%)	13.0	14.5	0.65	14.5	12.9	0.64	NS	+	NS
CD4 (%)	6.5	7.4	0.39	7.3	6.6	0.38	NS	NS	NS
CD8 (%)	5.3	5.0	0.32	4.9	5.4	0.32	NS	NS	+
CD4/CD8	1.3	1.5	0.12	1.4	1.4	0.12	NS	NS	NS
γδ (%)	1.4	1.7	0.11	1.6	1.5	0.11	+	NS	NS

NS ( $P > 0.05$ )+( $P < 0.1$ )

## DISCUSSION

The main findings of this study were that medium-term reduction in feeding and deprivation of lying failed to consistently affect lymphocyte proliferative capacity or population dynamics in lactating dairy cows. This raises the possibility that either the stressors were insufficient in magnitude to impact upon the immune system of the animals, or that such stressors do not contribute to altered immune function.

Deprivation of lying has been utilised previously for studying stress responses in dairy cows. Munksgaard and Simonsen (1996) deprived housed dairy cows of lying for two 7-hour periods each day for eight weeks. The lying deprivation in the present study represented a stronger (albeit shorter) challenge to the animals, as the total lying time for deprived cows was approximately 4 hours per day compared with 9.3 hours in the study by Munksgaard and Simonsen (1996). In the current study, the unrestricted animals had a total lying time of 9 hours, which is similar to other observations of 8.6 hours (Singh *et al.*, 1993) 10 hours (Webster, 1986; Leonard *et al.*, 1996), 11.6 hours (Arave *et al.*, 1991) and 11.8 hours (Sahara *et al.*, 1990). These shorter lying times may be associated with the management systems used. It would appear therefore, that the reduction in lying in this study represented a moderately strong challenge to the animals, although the lying deprived cows remained bright and healthy, and those that were also feed restricted were observed to always eat newly-offered feed following the removal of the girth straps, before choosing to lie down.

Although under-nourishment in animals can be a potent cause of immunosuppression (Filteau *et al.*, 1993), the duration and level of feed restriction of the cows in this study was not likely to be sufficiently severe as to reduce immunity by itself. Under-nutrition-induced immunosuppression is thought to result from a decreased availability

of amino acids and energy substrates to the cells of the immune system, a lack of micro-nutrients such as zinc, or the activation of the adrenal cortex to secrete elevated levels of glucocorticoid hormones. However, stress-induced glucocorticoid increases are not always responsible for concurrent changes in immune function (Filteau *et al.*, 1993; Minton *et al.*, 1994), and mean glucocorticoid concentrations in cattle often return to basal levels in cattle despite the presence of chronically-acting stressors (Ladewig and Smidt, 1989). Furthermore, it is unlikely that an essential body system such as immunity would be markedly compromised by the feeding regime used in the study, while less essential functions such as lactation continued.

In this study, the failure to demonstrate changes in lymphocyte proliferation in response to stressors of approximately one week duration contrasts with the results of previous studies examining more acute stressors. Blecha and Minocha (1983) recorded reduced proliferation in lymphocytes from cattle following 60 minutes of stressful exercise. A similar finding was reported by Kelley *et al.*, (1981) after transporting cattle by road for 480 km. Stressors of duration similar to that used in the present study appear to exert less readily determinable effects on immunity than acute events. In rats, one week of social stress reduced lymphocyte proliferation and both CD8 and CD4 subtypes in submissive, but not subdominant animals (Stefanski, 1998). It appears likely that, in cattle, lying deprivation and feed restriction such as used in the present study, do not impact measurably on cell-mediated immunity. Although integrated models of stress in animals include the downstream deleterious consequences of stressors in terms of reduced immune function and increased susceptibility to disease (Moberg, 1985), moderate stressors of medium duration may not be sufficiently severe to induce these effects.

In conclusion, this study indicates that lymphocyte function in lactating cows may not be compromised by medium term restrictions in feeding and lying, and that any stress-induced decreases in cell-mediated immunity which occur in practice, are the result of more severe, or prolonged, conditions.

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