

Lymphocyte Functions in Overconditioned Cows Around Parturition*

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ABSTRACT

The objective of the study was to evaluate the relationships between body condition and lymphocyte functions in periparturient dairy cows. Thirty days before expected calving, 21 Holstein cows were categorized as thin ($n = 6$), medium ($n = 8$), or overconditioned ($n = 7$) based on body condition score (BCS). Blood samples were collected on 21, 14, 7, and 3 d before calving and on d 3, 7, 14, 21, 28, and 35 after parturition. An aliquot of blood was used to determine plasma nonesterified fatty acids (NEFA) and glucose. At 14 and 7 d before, and 14 and 35 d after calving, a second aliquot of blood was used to assess peripheral blood mononuclear cell (PBMC) functions: DNA synthesis, immunoglobulin (Ig) M, and interferon-gamma (IFN- γ) secretion after mitogen stimulation. During the experiment, all 21 cows showed a decline in BCS. Overconditioned cows lost significantly more BCS than thin cows. After calving, overconditioned cows had higher plasma NEFA compared with thin and medium cows. Conversely, plasma glucose never differed between the 3 categories of cows. Regardless of BCS, DNA synthesis and IgM secretions were significantly lower in PBMC isolated on 7 d before calving compared with those recorded 14 and 35 d after parturition. Conversely, PBMC from the 21 cows did not show any change of IFN- γ secretion during the experimental period. Taking into consideration the BCS categories, PBMC isolated from overconditioned cows presented lower IgM secretion compared with thin cows on d 14 and 35 after calving. Furthermore, PBMC isolated from overconditioned cows secreted less IFN- γ compared with thin and medium cows on d 7 before calving. The DNA synthesis of PBMC stimulated with the 3 mitogens did not differ between the 3 categories of cows. In conclusion, immunodepression occurring in cows around calving would be particularly evident in overconditioned cows.

(Key words: periparturient cow, body condition score, lymphocyte function)

Abbreviation key: BrdU = 5-bromo-2'-deoxyuridine, ECM = enriched culture medium, PBMC = peripheral blood mononuclear cells, PWM = pokeweed mitogen.

INTRODUCTION

Several authors report that periparturient dairy cows are characterized by immunodepression (Mallard et al., 1998; Kehrl et al., 1999). Other authors specified that immunoresponsiveness decreases gradually in the prepartum period and reaches its minimum expression immediately before calving (Saad et al., 1989; Wagter et al., 1996). In dairy cows, the periparturient period is also characterized by profound endocrine and metabolic changes (Goff and Horst, 1997), which, according to some authors, may partially explain immunosuppression (Kaneene et al., 1997; Lacetera et al., 2004a).

With regard to lymphocyte functions, a series of studies (Targowski and Klucinski, 1983; Franklin et al., 1991; Suriyasathaporn et al., 1999) tested the hypothesis that hyperketonemia may in part explain the immunosuppression in the peripartum period. However, those studies provided conflicting results. Negative effects of ketones on functional activities of neutrophils have instead been reported in sheep and cows (Hoeben et al., 1997, 1999, 2000; Sartorelli et al., 1999, 2000; Suriyasathaporn et al., 1999). In particular, Hoeben et al. (2000) described negative relationships between neutrophil functions and plasma concentrations of BHBA and NEFA, and indicated plasma concentrations of these metabolites as possible diagnostic markers of impaired neutrophil function around calving. In a previous study carried out in ewes, we found negative relationships between cellular and humoral immunity and plasma concentrations of NEFA or BHBA (Lacetera et al., 2001). Furthermore, we found that addition of NEFA to culture medium was responsible for alteration of lymphocyte functions in sheep and cows (Lacetera et al., 2002, 2004a).

Previous studies carried out in other species demonstrated that obesity and weight loss are associated with dysfunction of the immune system (Samartín and Chandra, 2001). With regard to cows, no data have been found on relationships between obesity or overconditioning and lymphocyte functions, whereas overconditioning was associated with reduction of reproductive efficiency (Gil-

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Table 1. Ingredients and nutrients composition of diets fed during the experimental period (DM basis).

Ingredients (%)	Far-off dry cows	Close-up dry cows	Lactation cows
Corn silage	14.4	20.0	28.0
Triticale-grass silage	2.9	4.0	5.6
Alfalfa hay	5.2	7.3	10.2
Rye-grass hay	51.8	33.0	6.1
Corn ground	6.2	8.6	12.2
Oat ground	7.2	10.0	14.1
Soybean meal	5.5	7.5	10.7
Cotton seed	3.4	4.8	6.5
Dry beet pulp	2.6	3.6	5.1
Buffer ¹	0.5	0.7	0.9
Vitamin-mineral premix ²	0.3	0.5	0.6
Nutrient composition			
NE _L , Mcal/kg	1.29	1.41	1.62
Crude protein, %	12.70	13.70	15.60
NDF, %	43.10	40.00	36.40

¹A mixture of 33.3% CaCO₃, 31.7 Ca₃(PO₄)₂, 16.7% MgO, 16.6% NaHCO₃, and 1.7% ZnSO₄.

²Contained per kilogram: 6,000,000 IU of vitamin A; 600,000 IU of vitamin D₃; 7000 mg of vitamin E; 5000 mg of vitamin PP; 300 mg of vitamin B₁; 100 mg of vitamin B₂; 10,000 mg of choline chloride; 2 mg of vitamin B₁₂; 10,000 mg of Fe; 2,500 mg of Cu; 20,000 mg of Mn; 100 mg of Mo; 100 mg of Co; 800 mg of I; 50,000 mg of Zn; and 100 mg of Se.

lund et al., 2001) or alteration of liver functions (Drackley et al., 2001).

The present *ex vivo* study was carried out to evaluate the relationships between body condition and lymphocyte functions in periparturient dairy cows.

MATERIALS AND METHODS

Animals

The study was carried out on 21 multiparous healthy Holstein cows in a commercial herd, which were selected according to BCS on d 30 before calving. Body condition score was established according to ADAS (1986) using a 5-point scale. Six cows were categorized as thin (BCS < 2.5), 8 as medium (2.6 < BCS < 3.5), and 7 as overconditioned (BCS ≥ 3.5). The 3 groups were homogeneous for parity, and examination of the herd books indicated that none of the selected cows had suffered from reproductive or health problems previously. The BCS was then established weekly until the 35th day after parturition. Cows were fed diets (for dry and lactating cows, Table 1) consisting of a base ration fed as a TMR given daily at 0930 h and offered *ad libitum* to achieve 5 to 10% refusals. The close-up diet was offered starting 10 d before the expected calving.

Measurements, Samplings, and Laboratory Analyses

Feeds were sampled and analyzed. Dry matter was determined by forced-air oven drying at 65°C to constant

weight. Crude protein was determined by macro-Kjeldahl method (AOAC, 1984). Ether extract and ash were determined according to AOAC methods (AOAC, 1984). Neutral detergent fiber was analyzed according to the method described by Goering and Van Soest (1970).

Blood samples were collected on 21, 14, 7, and 3 d prepartum (before expected calving), and on d 3, 7, 14, 21, 28, and 35 after parturition via jugular venipuncture, using evacuated glass tubes coated with sodium heparin. An aliquot of blood was used to determine NEFA (NEFA-C kit; Wako Fine Chemical Industries USA, Inc., Dallas, TX) and glucose (Instrumentation Laboratory, Lexington, MA). Fourteen and 7 d before, and 14 and 35 d after calving, a second aliquot of blood was used to assess DNA synthesis, IgM, and IFN- γ secretion in peripheral blood mononuclear cells (PBMC) stimulated with mitogens.

The DNA synthesis was evaluated as already described (Lacetera et al., 2001). Peripheral blood mononuclear cells were isolated by density gradient centrifugation. Blood diluted in RPMI-1640 medium containing 25 mM HEPES (Sigma, Milano, Italy) was layered over Ficoll-Paque PLUS (APB, Milano, Italy) and centrifuged (600 × *g* for 45 min at 20°C). The mononuclear cell band was recovered and washed twice in PBS using centrifugation (400 × *g* for 10 min at 4°C). Residual red blood cells were eliminated by hypotonic shock treatment using redistilled water. The PBMC recovery and viability were determined by hemocytometer count using the trypan blue exclusion method. Viability of PBMC typically exceeded 90%. After isolation, PBMC were resuspended at a concentration of 1 × 10⁶ cells/mL of RPMI-1640 enriched culture medium (ECM). The ECM comprised RPMI-1640 containing 25 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin, 100 μ g of streptomycin, and 0.25 μ g of amphotericin B/mL (Sigma). Triplicate cultures were assayed using 96-well tissue culture plates. Each well contained 1 × 10⁵ mononuclear cells in 100 μ L of ECM. Control wells contained 100 μ L of PBMC suspension without mitogens. Additional control wells were used that contained 100 μ L of ECM without cells or 100 μ L of PBMC suspension without the pyrimidine analog 5-bromo-2'-deoxyuridine (BrdU). An optimal concentration of phytohemagglutinin (2.5 μ g/mL), pokeweed mitogen (PWM, 1 μ g/mL), or concanavalin A (2.5 μ g/mL) (Sigma) was added to plates. Plates were incubated in an atmosphere of 95% air and 5% CO₂ for 48 h at 39°C. Afterwards, 100 μ M BrdU in 10 μ L of RPMI-1640 was added to each well, and plates were incubated for an additional 18 h. The DNA synthesis was quantified by an ELISA assay (Lacetera et al., 2002). The assay was performed with a commercial kit (APB), and was based on measurement of BrdU incorporated during DNA synthesis in prolifer-

ating cells. Values for DNA synthesis were expressed as optical density for test wells minus optical density for control wells that did not contain BrdU. Intraassay coefficient of variation was 5.9%.

Secretion of IgM was established by growing PWM-stimulated cells (1×10^6 cells/well) under the same conditions as described above. Concentration of PWM was 0.2 $\mu\text{g}/\text{mL}$. Cells were cultured in duplicate for 12 d in 24-well plates. After 4 d of incubation, 1 mL of RPMI-1640 with 10% heat-inactivated fetal bovine serum was added to each well. At the end of the incubation, supernatants were collected and stored at -20°C until analyzed. The IgM released in culture medium was quantified by capture ELISA (Lacetera et al., 2002). Plates were coated with rabbit affinity-purified antibodies against bovine IgM. The IgM were detected using a sheep anti-bovine IgM- μ chain specific, conjugated with alkaline phosphatase. Antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Antibody concentrations (ng/mL) were calculated using a computer program (Fulvio Montauti, Pisa, Italy). The 50% absorbance values of serial dilutions of supernatant were interpolated onto the linear portion of a standard curve obtained by using purified bovine IgM (VMRD, Pullman, WA).

Secretion of IFN- γ was established by growing concanavalin A-stimulated cells (1×10^6 cells/well) under the same conditions described above. Cells were cultured in duplicate for 72 h in 24-well plates. At the end of incubation, supernatants were collected and stored at -20°C until analyzed using a commercial kit (CSL, Victoria, Australia). The IFN- γ concentration (ng/mL) was established using the same computer program used for IgM quantification. The 50% absorbance values of serial dilutions of supernatant were interpolated onto the linear portion of a standard curve obtained using bovine rIFN- γ (S. Jones, CSL, Victoria, Australia).

Statistical Analyses

Data for all variables measured were analyzed as repeated measures using the MIXED procedure of SAS (SAS Institute, 1999). The model included fixed effects (group: thin, medium and overconditioned, and day from calving: -14, -7, 14, and 35), random effect (cow within group), and interactions (group \times day from calving).

Data were analyzed across sampling days relative to day of calving, with d 0 representing the day of calving. For each analyzed variable, cow within group was subjected to 3 covariance structures: compound symmetric, first-order autoregressive, and unstructured covariance. The covariance structure that had the largest Akaike's information criterion and Schwarz's Bayesian criterion was considered the most desirable analysis (Littell et al., 1998; Doepel et al., 2002). Least square means were

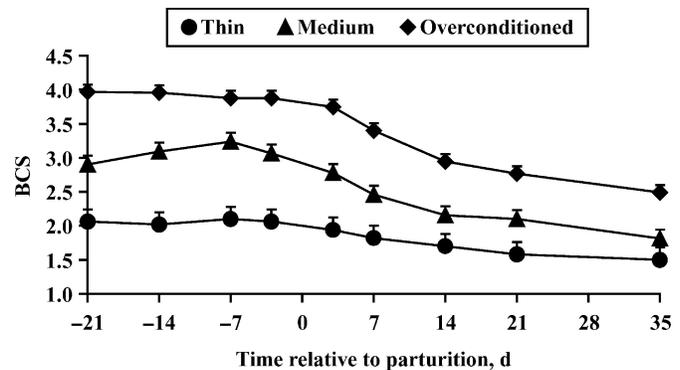


Figure 1. Body condition score in thin, medium, and overconditioned cows during the peripartum period. The difference in BCS loss between thin and overconditioned cows during the entire experimental period was statistically significant ($P < 0.01$). Values reported are LS means \pm SEM.

separated with the PDIF procedure of SAS (SAS Institute, 1999). Data are reported as least square means with standard errors of mean. Significance was declared at $P < 0.05$.

RESULTS

None of the cows developed clinical signs of diseases during the study.

During the experiment, all 21 cows showed a decline of BCS (Figure 1). However, the BCS loss was more pronounced in overconditioned cows. In detail, the loss of BCS was 0.6, 1.1, and 1.5 points in thin, medium, and overconditioned cows, respectively. The difference in BCS loss between thin and overconditioned cows during the entire experimental period was significant ($P < 0.01$).

Overconditioned cows showed higher plasma NEFA compared with thin and medium cows (Figure 2). In

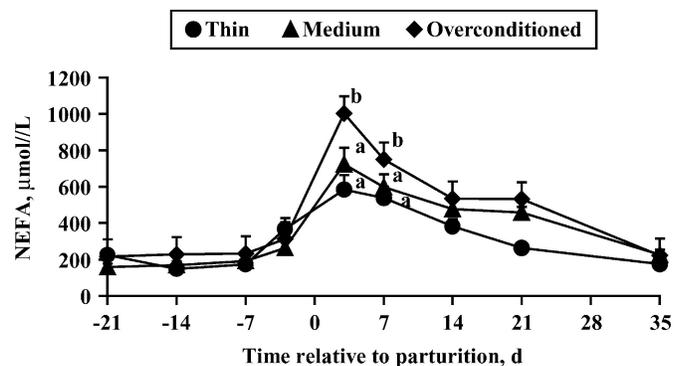


Figure 2. Plasma NEFA in thin, medium, and overconditioned cows during the peripartum period. Values with different letters differ significantly ($P < 0.01$). Values reported are LS means \pm SEM.

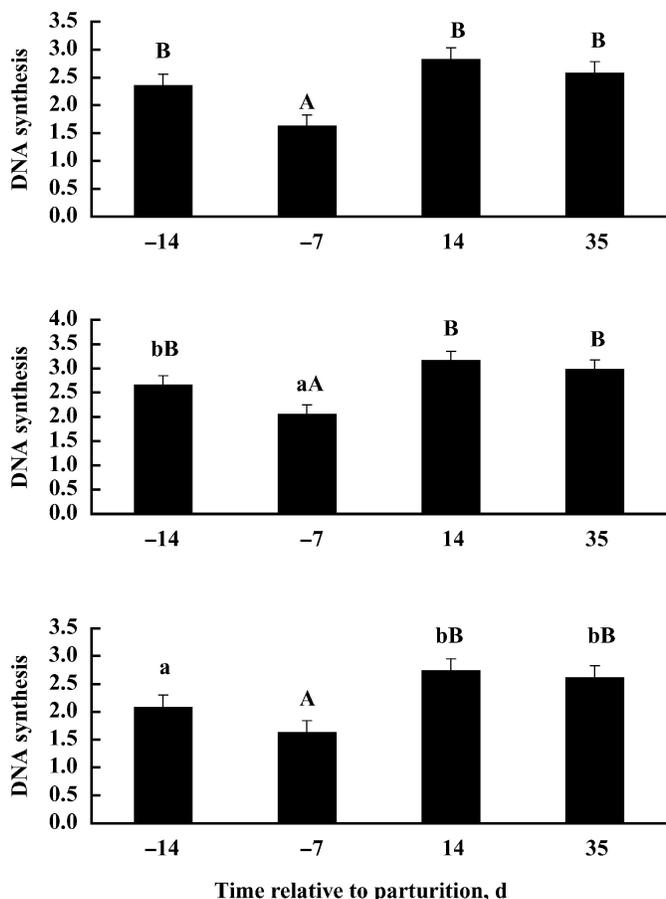


Figure 3. DNA synthesis in peripheral blood mononuclear cells stimulated with phytohemagglutinin (top), concanavalin A (middle), and pokeweed mitogen (bottom). Values reported are LS means \pm SEM of optical density. Columns with different letters differ significantly (a,b = $P < 0.05$; A,B = $P < 0.01$).

particular, on d 3 and 7 after parturition, plasma NEFA of overconditioned cows was higher ($P < 0.01$) than that recorded in the other 2 categories. Conversely, plasma glucose did not differ between the 3 categories of cows (not shown).

Regardless of the BCS categories, DNA synthesis and IgM secretion were significantly lower in PBMC isolated on 7 d before calving compared with those recorded 14 and 35 d following parturition (Figures 3 and 4). Conversely, PBMC from the 21 cows did not show any change of IFN- γ secretion during the experimental period (not shown).

Taking into consideration the BCS categories, PBMC isolated from overconditioned cows presented a lower IgM secretion compared with thin cows on d 14 and 35 after calving (Figure 5). Furthermore, PBMC isolated from overconditioned cows secreted less IFN- γ with respect to thin and medium cows on d 7 before calving (Figure 6). Conversely, the DNA synthesis of PBMC

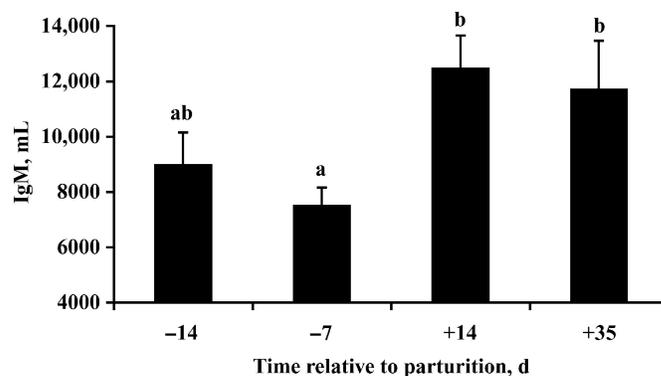


Figure 4. Immunoglobulin M secretion in peripheral blood mononuclear cells stimulated with pokeweed mitogen during the peripartum period. Values reported are LS means \pm SEM. Columns with different letters differ significantly ($P < 0.05$).

stimulated with the 3 mitogens did not differ between the 3 categories of cows.

Negative correlations were found between plasma NEFA and IgM, and IFN- γ in the group of overconditioned cows (Figures 7 and 8, respectively). In medium cows, a negative correlation was found only between plasma NEFA and IFN- γ (Figure 9). In other words, PBMC isolated from cows with the stronger mobilization of fat depots presented the lower response to mitogens in terms of IgM and IFN- γ secretions. No relationships were found between plasma NEFA and lymphocyte functions in thin cows, or between plasma glucose and lymphocyte functions in any of the 3 categories of cows.

DISCUSSION

Changes of BCS and plasma NEFA reported herein are consistent with those described by other authors in

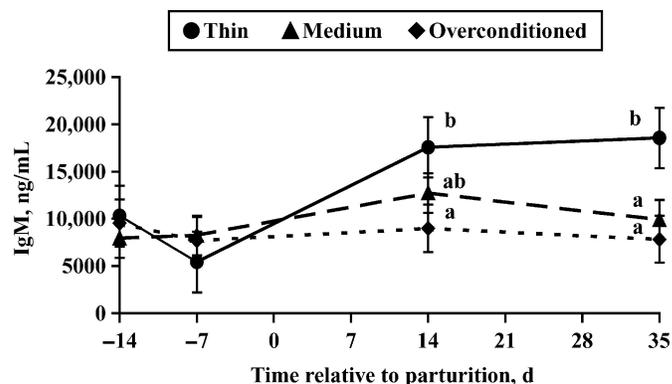


Figure 5. Immunoglobulin M secretion in peripheral blood mononuclear cells stimulated with pokeweed mitogen during the peripartum period in thin, medium, and overconditioned cows. Values reported are LS means \pm SEM. Values with different letters differ significantly ($P < 0.05$).

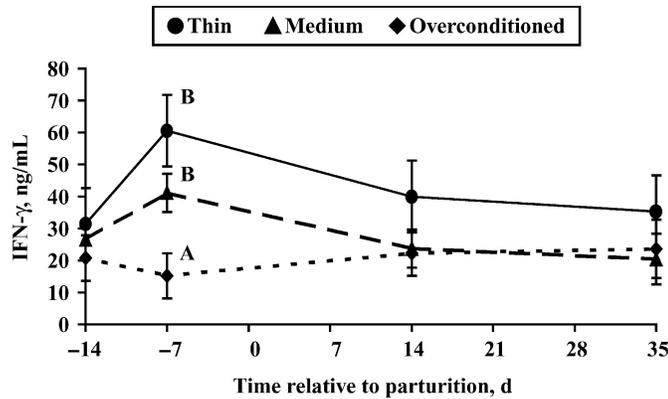


Figure 6. Interferon- γ (IFN- γ) secretion in peripheral blood mononuclear cells stimulated with concanavalin A during the peripartum period in thin, medium, and overconditioned cows. Values reported are LS means \pm SEM. Values with different letters differ significantly ($P < 0.01$).

periparturient dairy cows (Bertics et al., 1992; Grum et al., 1996; Bernabucci et al., 2004). Present results are also in line with previous findings, which indicated that mobilization of fat depots and increases of plasma NEFA are more intense in overconditioned cows (Van den Top et al., 1996; Rukkwamsuk et al., 1998, 2000). Reduction of DNA synthesis or IgM secretion immediately before calving is also consistent with previous data from cows or sheep (Saad et al., 1989; Lacetera et al., 2004b).

Neuroendocrine changes developing around parturition and nutritional imbalances that often occur in the same period have been evoked to justify the periparturient immunodepression in dairy cows (Goff and Horst, 1997; Mallard et al., 1998; Kehrli et al., 1999). The present study pointed out that BCS, intensity of lipomobilization, or increase of plasma NEFA affects lymphocyte functions in cows around calving. In particular, the ob-

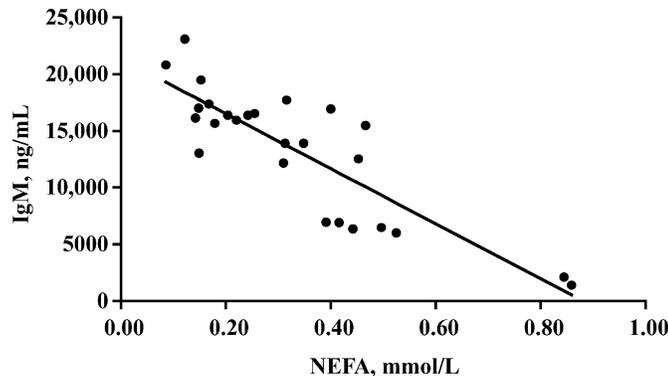


Figure 7. Relationship between plasma NEFA and IgM secretion in overconditioned cows. The solid line shows the regression $y = 21368.5 - 24246.6x$ ($r = -0.85$, $P < 0.01$).

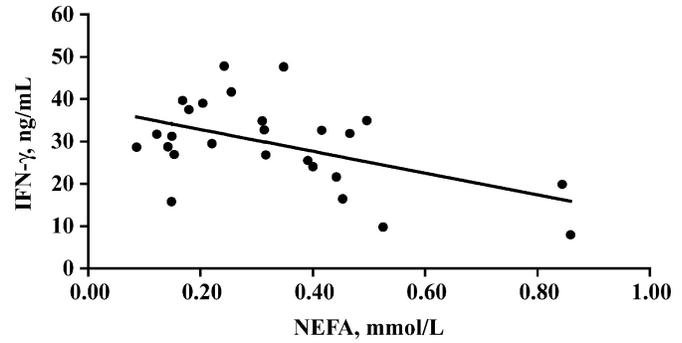


Figure 8. Relationship between plasma NEFA and IFN- γ secretion in overconditioned cows. The solid line shows the regression $y = 37.9 - 25.7x$ ($r = -0.50$, $P < 0.05$).

servation that PBMC isolated from overconditioned cows secrete less IgM and IFN- γ than those isolated from their medium or thin counterparts is novel and suggests new mechanisms to explain the immunodepression occurring around calving. In a recent review paper, Samartín and Chandra (2001) indicated that obesity is associated with a high incidence of infections and alteration of immunity in both laboratory animals and humans, and that this depends on several metabolic and endocrine factors (e.g., lipid and glucose metabolism, plasma concentration of leptin and insulin, etc.) related to overconditioning. Interestingly, a study carried out in premenopausal women (Nieman et al., 1996) indicated that it was weight loss that was associated with alterations of leukocyte functions, rather than obesity per se. Our results would indicate that in periparturient dairy cows, the alteration of lymphocyte functions in terms of IgM and IFN- γ secretions is proportional to the extent of condition loss as assessed by measuring changes of BCS and plasma NEFA. A previous study by Szuster-Ciesielska et al. (1995) pointed out a deficiency of IFN secretion in cows suffering from fat mobilization syn-

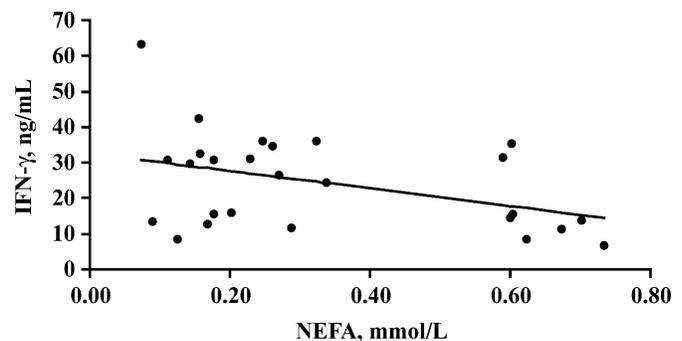


Figure 9. Relationship between plasma NEFA and IFN- γ secretion in medium cows. The solid line shows the regression $y = 32.6 - 24.8x$ ($r = -0.41$, $P < 0.05$).

drome. Wentink et al. (1997) documented that hepatic lipidosis due to intense lipomobilization was associated with impaired immunoreactivity of dairy cows. A prospective study by Kaneene et al. (1997) indicated that fat mobilization associated with energy deficit is related to increased risk of metritis and retained placenta in dairy cows. Hoeben et al. (2000) reported negative relationships between functions of bovine polymorphonuclear leukocytes and plasma concentrations of BHBA and NEFA. Finally, in a study carried out in periparturient sheep, we described negative relationships between humoral or cellular immunity and plasma concentrations of BHBA or NEFA (Lacetera et al., 2001).

A series of *in vitro* studies documented direct negative effects of NEFA on leukocyte functions in ruminants (Lacetera et al., 2002, 2004a). In detail, those studies were carried out using a mixture of fatty acids reflecting composition of NEFA in ruminant plasma, and at concentrations mimicking those occurring under conditions of intense lipomobilization. In sheep, that mixture of NEFA suppressed the ability of PBMC to proliferate and secrete IgM (Lacetera et al., 2002); in cows, the same mixture inhibited PBMC to synthesize DNA, and to secrete IgM and IFN- γ (Lacetera et al., 2004a).

Studies carried out in other species suggested several mechanisms through which fatty acids can interfere with lymphoid cell functions. These include perturbation of properties of cellular membranes, suppression of cytokine synthesis, formation of lipid peroxides, gene regulation, and induction of necrosis or apoptosis (De Pablo and de Cienfuegos, 2000). However, literature on this matter is often limited or conflicting, and in most cases, the molecular mechanisms involved in these processes have not been elucidated.

Finally, it is interesting to note that other authors hypothesized or demonstrated that BCS, intensity of lipomobilization, or increase of plasma NEFA may be involved in alteration of other physiological functions of periparturient dairy cows, namely reproduction (Gillund et al., 2001; Jorritsma et al., 2003a,b) and liver activities (Drackley et al., 2001).

The observation that plasma glucose did not differ between the 3 categories of cows would indicate that this parameter is not helpful in explaining the differences in immune function observed between thin, medium, and overconditioned cows. On the other hand, this hypothesis is supported by results from previous *in vitro* or *ex vivo* studies, which indicated that glucose concentrations in the culture media (Nonnecke et al., 1992) or plasma (Lacetera et al., 2001) did not significantly affect lymphocyte functions of cows and sheep, respectively. Franklin et al. (1991) reported that glucose concentrations had minimal effects on bovine lymphocyte proliferation *in vitro*.

CONCLUSIONS

Overconditioned cows have to be considered at high risk of infection because the intense lipomobilization taking place around calving is associated with alterations of lymphocyte functions.

The present study added further evidence to consider plasma NEFA as biochemical indicators of the immune reactivity in periparturient dairy cows. In particular, results of our correlation analysis would suggest that in transition cows with plasma NEFA not exceeding 600 $\mu\text{mol/L}$ (the thin cows of our study), there is no relationship between NEFA and lymphocyte function. These relationships are negative and increasingly significant only when plasma concentrations of these metabolites are beyond that limit (the medium and overconditioned cows in our study).

Further studies are being carried out to ascertain whether composition of plasma NEFA, in terms of proportions among single fatty acids, can interfere with immunoresponsiveness of dairy cows.

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