Effects of nonesterified fatty acids and β-hydroxybutyrate on functions of mononuclear cells obtained from ewes

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Objective—To assess the effects of nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) on functions of mononuclear cells obtained from ewes.

Animals—6 Sardinian ewes.

Procedure—Mononuclear cells were cultured with concentrations of NEFA (0, 15.6, 31.2, 62.5, 125, 250, 500, 1,000, or 2,000 μmol/L) and BHBA (0, 0.45, 0.9, 1.8, or 3.6 mmol/L). Concentrations of NEFA and BHBA were intended to mimic those of ketotic or healthy ewes, and NEFA and BHBA were tested alone and in combination. Synthesis of DNA was stimulated by use of concanavalin A (Con A) or pokeweed-mitogen (PWM). Secretion of IgM was stimulated by use of PWM.

Results—Synthesis of DNA stimulated by Con A and PWM was significantly inhibited by high concentrations of NEFA (≥ 250 μmol/L) or by a combination of high concentrations of NEFA (≥ 250 μmol/L) and all concentrations of BHBA (≥ 0.45 mmol/L). In contrast, DNA synthesis was not inhibited by low concentrations of NEFA (≤ 125 μmol/L) or by a combination of low concentrations of NEFA (≤ 125 μmol/L) and the lowest concentration of BHBA (0.45 mmol/L). Secretion of IgM was significantly inhibited by all concentrations of NEFA and by all combinations of NEFA and BHBA concentrations. When used alone, none of the concentrations of BHBA inhibited DNA synthesis or IgM secretion.

Conclusions and Clinical Relevance—Reduced immunoresponsiveness during ketosis is likely to be associated with an increase in plasma concentration of NEFA and not with an increase in plasma concentration of BHBA. (*Am J Vet Res* 2002;63:414–418)

Cows and ewes may develop acetonemia (ketosis), a metabolic disease commonly associated with pregnancy in ewes and lactation in cows.[1] Ketosis associated with pregnancy in sheep and lactation in cows is attributable to the high energy demands of pregnancy or lactation and is characterized by hypoglycemia and increased plasma concentrations of **nonesterified fatty acids (NEFA)** and ketone bodies.[2]

In sheep, clinical or subclinical ketosis during pregnancy is associated with impairment of cell-mediated and humoral immunity.[3,4] In cattle, epidemiologic studies[5,6] indicate that ketosis is associated with higher susceptibility to infections, which is believed to result from immunosuppression. Ketosis alters the immune response of ewes or causes higher incidence of infections in cows through mechanisms that have not yet been elucidated. However, it has been suggested that such consequences of ketosis may be associated with changes in metabolite concentrations in the blood.

Recently, we documented negative relationships between immunologic functions of ewes and plasma concentrations of NEFA or **β-hydroxybutyrate (BHBA)**, 1 of the most representative ketone bodies.[4] However, to our knowledge, in vitro studies have not been performed to assess the effects of NEFA or ketones on lymphocyte functions of sheep. Conversely, several in vitro studies[7-10] have been performed in cattle to ascertain whether high concentrations of ketones may explain the hypothesized impairment of the immune functions and the subsequent higher incidence of infections observed in ketotic cows. However, results of those studies are conflicting.

Therefore, the in vitro study reported here was performed to verify the effects of concentrations of NEFA or BHBA on DNA synthesis and IgM secretion of mitogen-stimulated mononuclear cells. Concentrations of NEFA or BHBA were designed to mimic those in healthy ewes and ewes affected with clinical or subclinical ketosis.

Materials and Methods

Animals—Six healthy nonpregnant, nonlactating, and nonketotic Sardinian ewes were used in the study. Each ewe was approximately 36 months old and weighed approximately 32 kg. Ewes were fed a diet consisting of forages and concentrate, which was formulated for sheep in accordance with nutritional requirements provided by Bocquier et al.[11] Blood samples were collected via jugular venipuncture, using evacuated glass tubes coated with sodium heparin (10 U of heparin/ml).

Preparation of media—A NEFA mixture was created that contained a combination of C16:0 (30%), C16:1 (5%), C18:0 (15%), C18:1 (45%), and C18:2 (5%), which reflected the composition of plasma NEFA reported for sheep.[12] This mixture was prepared, using the method of Strang et al.[13] Briefly, a stock solution of NEFA (88.8 m*M*) was prepared by dissolving fatty acids in 0.1N KOH at a molar ratio of 1:1.13 at 60 C. The NEFA-KOH solution, still at 60 C, was neutralized by addition of 1N HCl and diluted with sterile redistilled water. That solution was diluted in RPMI-1640, adjusted to pH 7.4, and sterilized by passage through a filter. This solution then was added to cell cultures grown in RPMI-1640 to reach final concentrations of 2,000, 1,000, 500, 250, 125, 62.5, 31.2, and 15.6 μmol/L of cell suspension.

The BHBA was dissolved in sterile PBS solution (final concentration, 360 mmol/L) and stored at –20 C until used.

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Before its addition to cell cultures, the BHBA solution was dissolved in RPMI-1640 culture medium to reach final concentrations of 3.6, 1.8, 0.9, and 0.45 mmol/L of cell suspension.

Evaluation of DNA synthesis—Heparinized blood was diluted 1:3 in sterile PBS solution. Twenty-four milliliters of diluted blood was layered over 16 ml of a nonionic synthetic polymer of sucrose[a] and centrifuged (600 × g for 45 minutes at 20 C). Mononuclear cells were removed from the interface and washed twice in PBS solution, using centrifugation (400 × g for 10 minutes at 4 C). Residual RBC were lysed by hypotonic shock treatment, using sterile redistilled water. Mononuclear cells were counted, using a hemacytometer. Cell viability was always > 95% as estimated by the trypan blue dye-exclusion method. Mononuclear cells were resuspended at a concentration of 2×10^6 cells/ml of RPMI-1640 culture medium enriched with 10% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, and 1% (vol:vol) antibiotics (10,000 U of penicillin/ml and 10 µg of streptomycin/ml).

Triplicate cultures were assayed, using 96-well tissue-culture plates. Each well contained 2×10^5 mononuclear cells in 100 µl of enriched culture medium. Control wells contained 100 µl of mononuclear cell suspension without mitogens, NEFA, or BHBA. Furthermore, as suggested by the manufacturer,[b] additional control wells were used that contained 100 µl of complete RPMI-1640 enriched medium without cells or 100 µl of mononuclear cell suspension without the pyrimidine analogue **5-bromo-2'-deoxyuridine (BrdU)**.

An optimal concentration of **pokeweed mitogen (PWM**; 0.4 µg/ml) or **concanavalin A (Con A**; 2.5 µg/ml) was added to plates containing various concentrations of NEFA (2,000, 1,000, 500, 250, 125, 62.5, 31.2, or 15.6 µmol/L) or BHBA (3.6, 1.8, 0.9, or 0.45 mmol/L) and to plates containing a combination of NEFA and BHBA (2,000 and 3.6, 1,000 and 1.8, 500 and 0.9, 250 and 0.45, 125 and 0.45, 62.5 and 0.45, 31.2 and 0.45, or 15.6 and 0.45 µmol of NEFA/L and mmol of BHBA/L, respectively).

Plates were incubated in an atmosphere of 95% air and 5% CO_2 for 48 hours at 37 C. We then added 10 µ*M* BrdU in 10 µl of RPMI-1640 to each well, and plates were incubated for an additional 18 hours. Culture medium then was removed from the tissue-culture plates, using centrifugation (300 × g for 10 minutes), and the tissue-culture plates were dried (60 C for 1 hour).

Cell proliferation was quantitated by use of an ELISA. The assay was performed, using a commercial kit[b] that is based on measurement of BrdU incorporated during DNA synthesis in proliferating cells. The ELISA was performed in accordance with the manufacturer's guidelines. Incubation time for peroxidase-labeled monoclonal anti-BrdU antibody was 90 minutes. Substrate was added and allowed to incubate for 15 minutes, and absorbance values then were obtained. Values for DNA synthesis were expressed as the **optical density (OD)** for test wells minus the OD for control wells that did not contain BrdU. Intra-assay coefficient of variation was 5.7%.

Evaluation of IgM secretion—Secretion of IgM was established by growing PWM-stimulated cells (2×10^6 cells/well) under the same conditions described previously. Cells were cultured in duplicate for 8 days in 24-well tissue-culture plates. At the end of the incubation period, plates were centrifuged (1,000 × g for 15 minutes), and supernatant was collected and stored at –20 C until analyzed. Analysis was performed within 2 weeks after supernatants were collected.

The amount of IgM released in culture medium was quantitated by use of a capture ELISA, performed as described elsewhere,[14] with minor modifications. Briefly, polystyrene microtitration plates were coated by incubation overnight with rabbit antiserum to sheep IgM[c] diluted at 1:3,000 in 50 m*M* carbonate-bicarbonate buffer, pH 9.4. After 3 washes with Tris-Tween-20 NaCl buffer (50 m*M* Tris-HCl [pH 7.4] containing 0.05% Tween-20 and 0.15*M* NaCl), saturation was carried out by use of a solution that consisted of the washing buffer with 3% bovine serum albumin. Serial dilutions of supernatant then were incubated in coated wells. The IgM were detected by use of an alkaline phosphatase-conjugated rabbit anti-sheep IgM polyclonal antibody.[d] The reaction was developed, using the substrate *p*-nitrophenyl phosphate, and the OD was measured on a spectrophotometer at a wavelength of 405 nm. Antibody concentrations were calculated, using a computer program.[e] The 50% absorbance values of serial dilutions of supernatant were interpolated onto the linear portion of a standard curve obtained by use of purified sheep IgM. Sheep IgM were purified from serum by initially removing IgG contamination, using affinity chromatography on protein G-sepharose,[f] and then by using thiophilic adsorption on a previously packed column.[g] Data were expressed as nanograms of IgM per milliliter of supernatant.

Statistical analysis—Data were reported as mean ± SEM. Data were analyzed by use of a 1-way ANOVA,[h] and effects were considered to be significant at a value of $P < 0.05$.

Results

Significant differences were not observed among results for mononuclear cells from the 6 ewes with regard to the pattern of influence exerted by NEFA and BHBA.

The DNA synthesis of Con-A- and PWM-stimulated mononuclear cells was significantly ($P < 0.001$) inhibited by high concentrations of NEFA (2,000, 1,000, 500, or 250 µmol/L; **Fig 1**) and by combinations of high concentrations of NEFA (2,000, 1,000, 500, or 250 µmol/L) and BHBA (3.6, 1.8, 0.9, and 0.45 mmol/L; **Fig 2**). In contrast, DNA synthesis of mitogen-stimulated mononuclear cells was not inhibited by low concentrations of NEFA (125, 62.5, 31.2, or 15.6 µmol/L) or by combinations of these

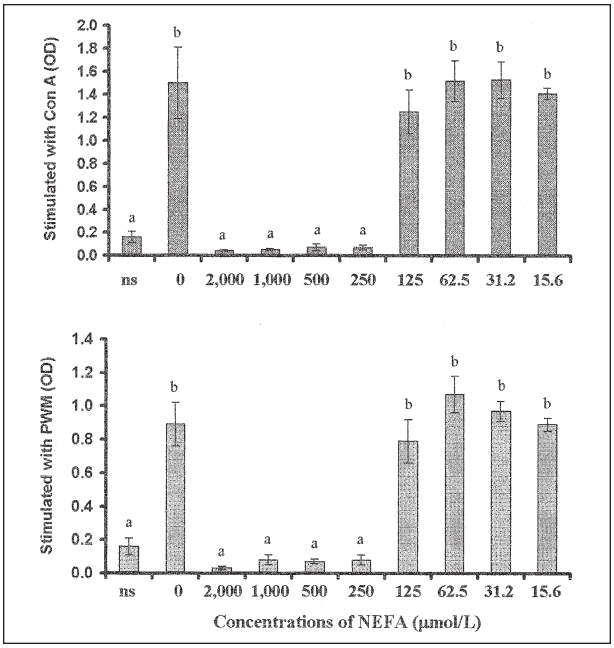

Figure 1—Effects of various concentrations of nonesterified fatty acids (NEFA) on DNA synthesis in ovine mononuclear cells stimulated with concanavalin A (Con A; top) and pokeweed mitogen (PWM; bottom). Values reported are mean ± SEM optical density (OD). [a,b]Within each panel, bars with different letters differ significantly ($P < 0.001$). ns = Not stimulated.

low concentrations of NEFA and 0.45 mmol of BHBA/L. In contrast to the effects of NEFA, none of the concentrations of BHBA alone inhibited DNA synthesis of mitogen-stimulated mononuclear cells (**Fig 3**).

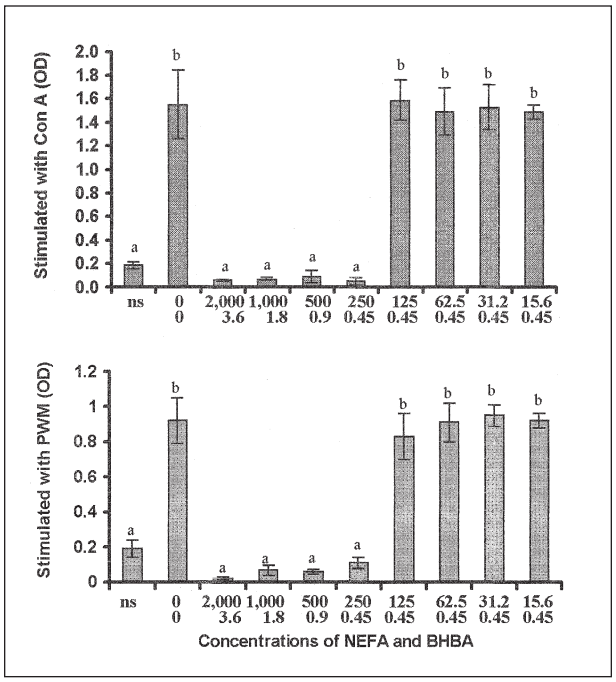

Figure 2—Effects of combinations of various concentrations of NEFA and β-hydroxybutyrate (BHBA) on DNA synthesis in ovine mononuclear cells stimulated with Con A (top) and PWM (bottom). Values reported are mean ± SEM OD. The top row of numbers on the x-axis indicates concentrations of NEFA (μmol/L), and the bottom row of numbers on the x-axis indicates concentrations of BHBA (mmol/L). *See* Figure 1 for key.

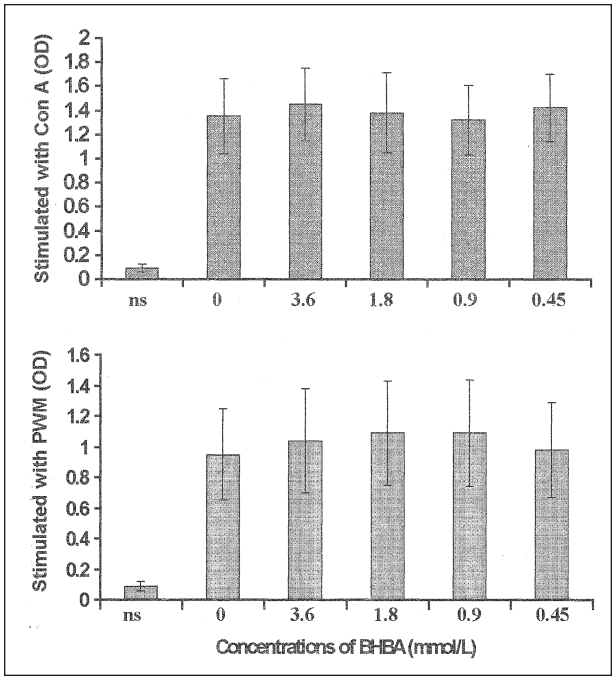

Figure 3—Effects of various concentrations of BHBA on DNA synthesis in ovine mononuclear cells stimulated with Con A (top) and PWM (bottom). Values reported are mean ± SEM OD.

The IgM secretion of PWM-stimulated mononuclear cells was significantly inhibited by all concentrations of NEFA (**Fig 4**) and by all combinations of the various concentrations of NEFA and BHBA (**Fig 5**). In particular, inhibition was especially evident for concentrations of NEFA between 2,000 and 125 μmol/L, alone or in combination with BHBA. Conversely, IgM secretion was not affected by the addition to culture media of any of the 4 concentrations of BHBA tested in this study (**Fig 6**).

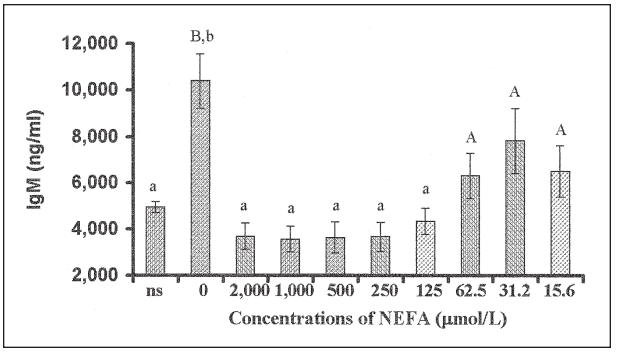

Figure 4—Effects of various concentrations of NEFA on IgM secretion in ovine mononuclear cells stimulated with PWM. Values reported are mean ± SEM. [a,b]Bars with different letters differ significantly ($P < 0.001$). [A,B]Bars with different letters differ significantly ($P < 0.05$).

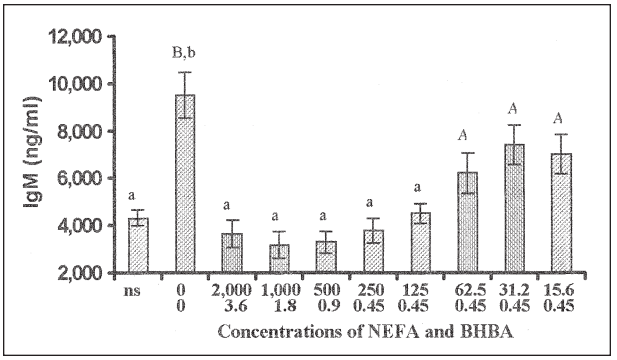

Figure 5—Effects of combinations of various concentrations of NEFA and BHBA on IgM secretion in ovine mononuclear cells stimulated with PWM. Values reported are mean ± SEM. The top row of numbers on the x-axis indicates concentrations of NEFA (μmol/L), and the bottom row of numbers on the x-axis indicates concentrations of BHBA (mmol/L). *See* Figure 4 for key.

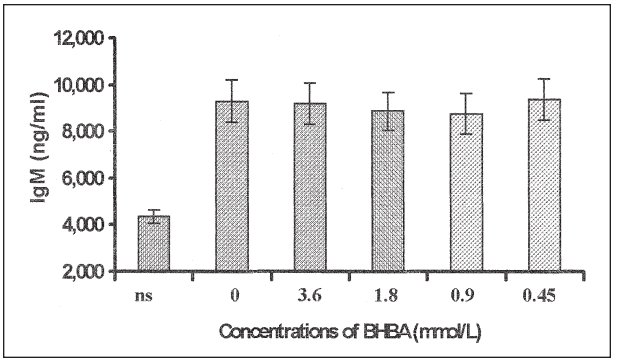

Figure 6—Effects of various concentrations of BHBA on IgM secretion in ovine mononuclear cells stimulated with PWM. Values reported are mean ± SEM.

Discussion

Analysis of results of the in vitro study reported here indicated that the reduced immunoresponsiveness observed in ewes with ketosis is likely to be related to ketosis-associated changes in concentrations of blood metabolites. To our knowledge, there have not been any published reports concerning the combined effects of NEFA and BHBA or the effects of NEFA alone on lymphocyte functions of ruminants. In another study[4] that involved the use of sheep, we reported that in vitro DNA synthesis of mitogen-stimulated lymphocytes or in vivo production of antigen-specific antibodies were significantly reduced during subclinical ketosis and negatively associated with plasma concentrations of NEFA. With regard to cattle, Reid et al[15] reported that high fat content in the liver was associated with a reduction in the number of WBC in the peripheral circulation. Results of the in vitro study reported here indicated that alterations of lymphocyte functions observed by combining high concentrations of NEFA and BHBA are the same as those observed when lymphocytes are incubated with high concentrations of NEFA alone. These results also suggested that the high concentrations of NEFA recorded in ewes with ketosis have to be considered as 1 of the possible causes of the immunosuppression that are evident in such conditions.

With regard to laboratory animals and humans, the ability of lymphocytes to incorporate and use exogenously derived free fatty acids has been reported in numerous studies.[16-22] Authors of those studies described an inhibitory effect of fatty acids on several immune functions, including lymphocyte proliferation, antibody secretion, cytokine production, natural killer cell activity, phagocytosis and inflammatory response, and expression of cell surface markers. However, the mechanisms involved in modulation of the immune system by fatty acids have not been clearly elucidated. It is likely that cellular effects of fatty acids are attributable to partition of these highly hydrophobic molecules into cellular membranes with subsequent perturbation of their biophysical and functional properties.[23] Incorporation of fatty acids into the cellular membranes of lymphocytes can negatively influence the fluidity of the lipid bilayer and render the cell membrane more rigid, consequently resulting in possible alterations involving transportation of ions or substrates into the cells, activity of membrane-associated enzymes and receptors, and activity of lymphocyte-signaling pathways.[16,20,21]

Analysis of results of another study[17] suggests that the NEFA-related reduction of lymphocyte functions may also depend on suppression of cytokine synthesis. The mechanism involved in modification of cytokine synthesis attributable to fatty acids remains unclear, but a possible explanation could be found in regulation at the transcriptional level,[24] because fatty acids may inhibit cytokine mRNA production.

Additional mechanisms have been proposed to explain modulation of the immune system attributable to the effects of fatty acids. Among these are formation of lipid peroxides and eicosanoids, gene regulation, and apoptosis.[22,25,26] However, literature concerning these hypotheses is limited or conflicting, and in most cases, the molecular mechanisms involved in these processes have not been elucidated.

Data provided in the study reported here documented that NEFA can inhibit mitogen-dependent DNA synthesis and IgM secretion of sheep lymphocytes in a dose-related manner and that DNA synthesis and IgM secretion had differing sensitivity to concentrations of NEFA. In the conditions for our study, IgM secretion continued to be significantly inhibited by low concentrations of NEFA, that had ceased to affect the DNA synthesis. Regardless of the species, this observation is novel and not easy to explain. Currently, we can only hypothesize that such results may be related to the complexity of the antibody-secretion process, which requires proliferation of antibody-secreting cells, differentiation of those cells, secretion of a number of cytokines, and biochemical and biophysical interactions among various mononuclear cell types and lymphocyte subpopulations.[27] In our experiences, we have observed that addition of NEFA to cultures of mononuclear cells dramatically reduces secretion of interferon-γ a cytokine produced by helper-1 T cells that stimulates immunoglobulin light-chain synthesis.[28]

To our knowledge, effects of specific ketones on lymphocyte functions in sheep have not been reported. Recently, we reported that the ability of sheep lymphocytes to synthesize DNA after mitogen stimulation was significantly reduced during subclinical ketosis and negatively associated with plasma concentrations of BHBA.[4] However, results of the in vitro study reported here indicated that alterations of lymphocyte functions observed by combining high concentrations of NEFA and BHBA are not observed when lymphocytes are incubated with only BHBA. Therefore, high concentrations of BHBA recorded in ewes with ketosis should not be considered directly responsible for the immunosuppression evident during such conditions.

Relationships between ketone bodies and functions of mononuclear cells have been widely investigated in cattle; however, results of those studies are conflicting. In several studies,[7,8,29,30] it was reported that mitogenic response of blood or milk lymphocytes was negatively affected by toxic and subtoxic concentrations of ketone bodies in the culture media. Other authors[31] also suggested that high concentrations of ketone bodies may explain suppression of interferon production observed in ketotic cows. Conversely, some authors[9,10,32,33] did not observe any effect of various concentrations of ketone bodies added to culture media on lymphocyte proliferation or IgM secretion of mitogen-stimulated mononuclear cells. Franklin et al[9] suggested that such discrepancies among results in cattle may be attributable to several factors, including age of the animals, type and concentration of ketone bodies, duration and temperature used for incubation of cultures, or differences in culture media.

Analysis of results of the in vitro study reported here indicated that alterations of lymphocyte functions observed when combining high concentrations of NEFA and BHBA depend on the concentration of NEFA and that decreased efficiency of the immune system observed in ketotic ewes is likely to be associated with

the increase in plasma concentration in NEFA and not with the increase in plasma concentration of BHBA. Finally, analysis of our data also suggested that plasma NEFA may represent fine biochemical indicators of the reactivity of the immune system of sheep and that intense lipomobilization, even when not associated with hyperketonemia, has to be considered as potentially detrimental for the immune responses of sheep.

[a]Ficoll-Paque Plus, APB, Milano, Italy.
[b]Biotrak, APB, Milano, Italy.
[c]Rabbit antiserum to sheep IgM (μ chain), ICN Biomedicals Inc, Costa Mesa, Calif.
[d]Rabbit anti-sheep IgM-μ chain specific, Bethyl Laboratories, Montgomery, Tex.
[e]Reglin, Fulvio Montauti, Pisa, Italy.
[f]Protein G sepharose 4 fast flow lab packs, APB, Milano, Italy.
[g]HiTrap IgM purification, APB, Milano, Italy.
[h]Instat, GraphPad Software Inc, San Diego, Calif.

References

1. Kaneko JJ. Carbohydrate metabolism and its diseases. In: Kaneko JJ, Harvey JW, Bruss ML, eds. *Clinical biochemistry of domestic animals*. San Diego: Academic Press Inc, 1997;79:45–81.

2. Schäfer M. Disorders in saccharide metabolism. In: Vrzgula L, ed. *Metabolic disorders and their prevention in farm animals*. Amsterdam: Elsevier Science Publishers, 1991;63–83.

3. Singh SK, Prasad MC, Singh N. Influence of pregnancy toxaemia on immune status of sheep. *Indian J Vet Pathol* 1993;17:106–109.

4. Lacetera N, Bernabucci U, Ronchi B, et al. Effects of subclinical pregnancy toxemia on immune responses in sheep. *Am J Vet Res* 2001;62:1020–1024.

5. Erb HN, Grohn YT. Epidemiology of metabolic disorders in the periparturient dairy cow. *J Dairy Sci* 1988;71:2557–2571.

6. Duffield T. Subclinical ketosis in lactating dairy cattle. *Vet Clin North Am Food Anim Pract* 2000;16:231–253.

7. Targowski SP, Klucinski W. Reduction in mitogenic response of bovine lymphocytes by ketone bodies. *Am J Vet Res* 1983;44:828–830.

8. Targowski SP, Klucinski W, Littledike ET, et al. Suppression of mitogenic response of bovine lymphocytes during experimental ketosis in calves. *Am J Vet Res* 1985;46:1378–1380.

9. Franklin ST, Young JW, Nonnecke BJ. Effects of ketones, acetate, butyrate and glucose on bovine lymphocyte proliferation. *J Dairy Sci* 1991;74:2507–2514.

10. Quigley JD, Rejman JJ, Martin KR. Response of peripheral blood mononuclear cells to mitogenic in young calves. *J Dairy Sci* 1994;77:259–263.

11. Bocquier F, Theriez M, Prache S, et al. Alimentation des ovins. In: Jarrige R, ed. *Alimentation des bovins, ovins and caprins*. Paris: Institut National de la Recherche Agronomique, 1988;249–280.

12. Noble RC, Steele W, Moore J. The plasma lipids of the ewe during pregnancy and lactation. *Res Vet Sci* 1971;12:17–53.

13. Strang BD, Bertics SJ, Grummer RR, et al. Effect of long-chain fatty acids on triglyceride accumulation, gluconeogenesis, and ureagenesis in bovine hepatocytes. *J Dairy Sci* 1998;81:728–739.

14. Scapigliati G, Romano N, Picchietti S, et al. Monoclonal antibodies against sea bass *Dicentrarchus labrax* (L.) immunoglobulins: immunolocalisation of immunoglobulin-bearing cells and applicability in immunoassays. *Fish Shellfish Immunol* 1996;6:383–401.

15. Reid IM, Collins RA, Dew AM, et al. Immune competence of dairy cows with fatty liver. In: Wensing T, ed. *Production disease in farm animals*. Wageningen, Holland: Wageningen Pers, 1983;191–194.

16. Calder PC, Newsholme EA. Influence of antioxidant vitamins on fatty acid inhibition of lymphocyte proliferation. *Biochem Mol Biol Int* 1993;29:175–183.

17. De Pablo MA, de Cienfuegos A. Modulatory effects of dietary lipids on immune system functions. *Immunol Cell Biol* 2000;78:31–39.

18. Pourbohloul S, Mallett GS, Buttke TM. Inhibition of lymphocyte proliferation by free fatty acids III. Modulation of thymus-dependent immune responses. *Immunology* 1985;56:659–666.

19. Chow SC, Ansotegui IJ, Jondal M. Inhibition of receptor-mediated calcium influx in T cells by unsaturated non-esterified fatty acids. *Biochem J* 1990;267:727–732.

20. Richieri GV, Kleinfeld AM. Free fatty acids perturbation of transmembrane signaling in cytotoxic T lymphocytes. *J Immunol* 1989;143:2302–2310.

21. Breittmayer JP, Pelassi C, Cousin J, et al. The inhibition by fatty acids of receptor-mediated calcium movements in Jurkat T-cells is due to increased calcium extrusion. *J Biol Chem* 1993;268:20812–20817.

22. Stulnig TM, Berger M, Roden M, et al. Elevated serum free fatty acid concentrations inhibit T lymphocyte signaling. *FASEB J* 2000;14:939–947.

23. Anel A, Richieri GV, Kleinfeld AM. Membrane partition of fatty acids and inhibition of T cell function. *Biochemistry* 1993;32:530–536.

24. Robinson DR, Urakaze M, Huang R, et al. Dietary marine lipids suppress continuous expression of interleukin-1β gene transcription. *Lipids* 1996;31(suppl):23–31.

25. Carratelli CR, Nuzzo I, Vitiello T, et al. The effect of dietary lipid manipulation on murine splenic lymphocytes apoptosis and heat shock protein over expression. *FEMS Immunol Med Microbiol* 1999;46:619–624.

26. De Pablo MA, Susin SA, Jacotot E, et al. Palmitate induces apoptosis via a direct effect on mitochondria. *Apoptosis* 1999;4:81–87.

27. Franklin ST, Young JW, Nonnecke BJ. Proliferation and phenotype of bovine mononuclear leukocytes in cultures stimulated by pokeweed mitogen. *J Dairy Sci* 1994;77:3592–3600.

28. Tizard IR. Interferons. In: Myers MJ, Murtaugh MP, eds. *Cytokines in animal health and disease*. New York: Marcel Dekker, 1995;24:1–57.

29. Klucinski W, Miernik-Degorska A, Degorska S, et al. Effect of ketone bodies on mitogenic response of bovine milk lymphocytes. *Zentralbl Veterinarmed [A]* 1988;35:626–631.

30. Sato S, Suzuki T, Okada K. Suppression of mitogenic response of bovine peripheral blood lymphocytes by ketone bodies. *J Vet Med Sci* 1995;57:183–185.

31. Szuster-Ciesielska A, Filar J, Kandefer-Szerszen M. Depression of interferon production in leukocytes of cows with fat mobilization syndrome. *Arch Immunol Ther Exp* 1995;43:61–65.

32. Nonnecke BJ, Kehrli ME Jr. Isolation of mononuclear cells from bovine milk by continuous flow and density gradient centrifugation: response of cells to mitogens. *Am J Vet Res* 1985;46:1259–1262.

33. Nonnecke BJ, Franklin ST, Young JW. Effects of ketones, acetate, and glucose on in vitro immunoglobulin secretion by bovine lymphocytes. *J Dairy Sci* 1992;75:982–990.