Effects of subclinical pregnancy toxemia on immune responses in sheep

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Objective—To determine the effects of moderate feed restriction, single or twin pregnancy, and subclinical pregnancy toxemia (PT) on immune responses of ewes.

Animals—16 Sardinian ewes.

Procedure—Six weeks before lambing, ewes were assigned to 1 of 2 groups (n = 8/group) matched for number of fetuses, body condition score, and plasma glucose, non-esterified fatty acids (NEFA), and β-hydroxybutyrate (BHBA) concentrations, and feed intake was restricted for 1 of the groups. Cell-mediated immunity was evaluated in vivo and in vitro. Humoral immunity was evaluated in vivo by determining production of IgG antibodies against keyhole limpet hemocyanin (KLH).

Results—Four ewes developed subclinical PT (plasma BHBA concentration > 0.86 mmol/L without any clinical signs of disease). Whether feed was restricted and type of pregnancy (single vs twin) did not have any significant effects on cell-mediated and humoral immune responses. Ewes with subclinical PT had significantly lower in vitro proliferation of phytohemagglutinin-stimulated peripheral blood mononuclear cells and significantly lower values for KLH-specific IgG than did healthy ewes. Plasma BHBA and NEFA concentrations were negatively correlated with in vitro proliferation of peripheral blood mononuclear cells; plasma NEFA concentration was negatively correlated with values for KLH-specific IgG.

Conclusions and Clinical Relevance—Results suggest that subclinical PT may be associated with impairments in cell-mediated and humoral immune responses in sheep. *Am J Vet Res* 2001;62:1020–1024)

In countries with intensive sheep breeding, especially during winter, more than 20% of the ewes in the late stages of pregnancy may develop a pregnancy-associated ketosis known as **pregnancy toxemia (PT)**.[1,a] The condition may be clinical or subclinical and is more common in sheep carrying 2 or more fetuses[2] and sheep that are undernourished.[3] Similar to bovine ketosis, a lactation-associated ketosis, PT is related to disturbances in glucose homeostasis and excessive lipid mobilization[4-6] and is characterized by hypoglycemia, high plasma concentrations of free fatty acids, and hyperketonemia.[3,7,8,a]

In cows, clinical or subclinical ketosis increases.[9-13] This effect has been ascribed to immunodepression[12]; however, results of in vivo studies on immunoresponsiveness of cows with clinical or subclinical ketosis are limited, and those of in vitro studies are conflicting. In contrast, a study[14] of Muzzaffarnagari ewes with experimentally induced clinical PT found that the disease was associated with significant depression in cell-mediated immunity. Two other studies[12,15] reported impairments in particle uptake, chemotaxis, and bactericidal activity by sheep neutrophils incubated with concentrations of **β-hydroxybutyrate (BHBA)** mimicking those in sheep with mild or severe ketosis.

To our knowledge, however, no studies of the effects of subclinical PT on immune responses of sheep have been published. Similarly, no information is available on whether the 2 principal predisposing factors for PT—type of pregnancy (single vs twin) and undernutrition—have any effects on the immune responses of ewes. The purposes of the study reported here, therefore, were to determine whether undernutrition, type of pregnancy, or subclinical PT had any effects on cell-mediated or humoral immunity in ewes in the late stages of pregnancy.

Materials and Methods

Animals—Sixteen healthy primiparous Sardinian ewes obtained from a commercial dairy sheep unit were used in the study. All of the ewes were approximately 13 months old at the start of the study; mean ± SD body weight was 38.1 ± 0.8 kg. The study was performed during the winter (November through January) at the experimental farm of the Università della Tuscia.

At the commercial dairy sheep unit, expected lambing dates of the ewes were synchronized by intravaginal implantation of progesterone-impregnated sponges. Ultrasonography was performed 35 days after natural mating to determine the number of fetuses. Ewes were transferred from the commercial unit to the experimental farm 8 weeks before expected lambing (mean ± SD of 7.9 ± 0.2 weeks before actual lambing), and were allowed a 2-week adaptation period at the experimental farm prior to the start of the study.

Experimental protocol—Ewes were assigned to 1 of 2 groups (n = 8/group) matched on the basis of number of fetuses, **body condition score (BCS)**, and plasma glucose, **non-esterified fatty acids (NEFA)**, and BHBA concentrations. Four ewes in each group were carrying 2 fetuses; the remaining 4 ewes in each group were each carrying only a single fetus. Body condition scores were assigned by a single individual, using established criteria.[16] Blood samples were collected by means of jugular venipuncture, anticoagulated with heparin (10 U/ml), and centrifuged at 3,500 × g for 15 minutes. Plasma was immediately analyzed to determine glucose, NEFA, and BHBA concentrations, as described.[17] Throughout the study, BCS were assigned, and plasma glucose, NEFA, and BHBA concentrations were measured weekly.

During the adaptation period, ewes were fed a ration developed to meet the published nutritional requirements of

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sheep.[18] The ration consisted of 0.3 kg of concentrate/head/d and a mixture of alfalfa silage (1.00 kg/head/d), alfalfa hay (0.60 kg/head/d), and Italian ryegrass hay (0.50 kg/head/d). After this time, ewes in group 1 were fed the same ration. Ewes in group 2 were subjected to moderate feed restriction and were given 70% of the concentrate (0.21 kg/head/d) and forages (0.62 kg of alfalfa silage/head/d, 0.36 kg of alfalfa hay/head/d, and 0.31 kg of Italian ryegrass hay/head/d) that they had voluntarily ingested during the adaptation period. Feed intake was restricted for these ewes in an attempt to increase the number of ewes that developed subclinical PT.[3]

Ewes in the 2 groups were fed from separate feed bunks. Forages were given at 8 AM; the concentrate was given at 9 AM and 1 PM. The ewes had free access to tap water. Daily intake of concentrate and forages was recorded during the adaptation period and the study.

In vivo evaluation of cell-mediated immunity—One week before expected lambing, cell-mediated immunity was evaluated in vivo by measuring the increase in double skinfold thickness after intradermal injection of 250 µg of **phytohemagglutinin (PHA)**,[b] as described.[19] The PHA was diluted in 0.1 ml of sterile **phosphate-buffered saline solution (PBSS)** and was injected in the middle of a 15 × 10-cm area of skin bordered by the coxal tuber, ischiatic tuber, and sacral vertebrae; hair was clipped from the skin prior to injection of PHA. An automatic syringe for intradermal injections[c] was used. Double skinfold thickness was measured, using a constant tension caliper,[d] before and 6, 12, 24, and 48 hours after the injection. Control injections of sterile PBSS were used to ensure that the response was specific to PHA. The PBSS and PHA injection sites were separated by approximately 10 cm.

In vitro evaluation of cell-mediated immunity—Cell-mediated immunity was evaluated in vitro by measuring proliferative responses of **peripheral blood mononuclear cells (PBMC)**. One week before expected lambing, prior to intradermal injection of PHA, jugular vein blood samples were collected from the ewes. Samples were anticoagulated with heparin (10 U/ml), and 6 ml of blood was diluted with 9 ml of RPMI-1640 medium containing 25 m*M* HEPES buffer.[b] Diluted blood (15 ml) was layered over 9 ml of ficoll[e] and centrifuged at 600 × g at 20 C for 45 minutes. The mononuclear cell band was recovered and washed twice in RPMI-1640 medium containing 25 m*M* HEPES buffer, followed by centrifugation at 400 × g at 4 C for 10 minutes. Residual RBC were lysed by hypotonic shock treatment, using sterile redistilled water. Recovery and viability of PBMC were determined by hemocytometer count, using the trypan blue exclusion method. Viability of PBMC typically exceeded 90%. The PBMC were resuspended at a concentration of 2×10^6 viable cells/ml in RPMI-1640 medium containing 25 m*M* HEPES buffer that was supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, 100 units of penicillin/ml, 100 µg of streptomycin/ml, and 0.25 µg of amphotericin B/ml. Time between blood collection and establishment of cultures was < 5 hours.

For determination of cell proliferation, 100 µl of PBMC was added in triplicate to wells on 96-well flat-bottom tissue culture plates.[f] The T-lymphocyte mitogen PHA[b] (2 µl) was added at a final concentration of 30 µg/ml, as results of preliminary experiments indicated that this concentration of PHA induced optimal responses under the culture conditions used. Control wells contained 100 µl of the PBMC suspension without PHA. As suggested by the manufacturer, additional control wells contained 100 µl of complete RPMI-1640 medium or 100 µl of the PBMC suspension without **5-bromo-2'-deoxyuridine (BrdU)**. Tissue culture plates were incubated for 48 hours at 37 C in an atmosphere of 95% air and 5% CO_2. Thereafter, 10 µM of the pyrimidine analogue BrdU[g] in 10 µl of RPMI-1640 was added to each well. After an additional 18 hours of incubation, culture medium was removed by centrifuging the tissue culture plates at 300 × g for 10 minutes. The tissue culture plates were then dried at 60 C for 1 hour. Cell proliferation was quantitated by use of an ELISA. The assay was performed with a commercial kit[g] based on measurement of BrdU incorporation during DNA synthesis in proliferating cells. Tests were performed according to the manufacturer's guidelines. The incubation time with peroxidase-labeled monoclonal anti-BrdU antibody was 90 minutes. Absorbance values were read with a microtitration plate spectrophotometer[h] after 15 minutes incubation with the substrate. Values for in vitro PBMC proliferation were expressed as the **optical density (OD)** for test wells minus the OD for control wells without BrdU.

The BrdU incorporation method was recently shown to provide results equivalent to those obtained with the [^{3}H]thymidine incorporation assay when used to evaluate proliferation of mitogen-stimulated sheep PBMC.[20] Previous studies[21-23] have also demonstrated the validity of this method for evaluating PBMC proliferative responses in other species. The intra-assay coefficient of variation for the assay was 6.2%.

Evaluation of humoral immunity—Four and 2 weeks before expected lambing, ewes were inoculated intramuscularly with 500 µg of **keyhole limpet hemocyanin (KLH)**[b] dissolved in 0.5 ml of sterile PBSS and emulsified in 0.5 ml of **incomplete Freund adjuvant (IFA)**.[b] One group-1 and 1 group-2 ewe were sham immunized with 0.5 ml of sterile PBSS emulsified in IFA as controls.

Blood samples were collected before the first inoculation and at weekly intervals for the next 3 weeks. Blood samples were collected by means of jugular venipuncture into containers without anticoagulants and allowed to clot. Samples were then centrifuged for 15 minutes at 3,500 × g, and serum was harvested and stored at –20 C until analyzed.

Serum IgG specific for KLH was quantitated by use of an indirect ELISA, performed as described, with minor modifications.[24] Briefly, polystyrene microtitration plates[i] were coated overnight with KLH at a concentration of 10 µg/ml in carbonate-bicarbonate buffer, pH 9.4. After saturation with a solution of 3% bovine serum albumin[b] in wash buffer, serial dilutions of the serum samples were incubated in coated wells. Serum IgG antibodies were detected by use of an AP-conjugated donkey antisheep IgG polyclonal antibody.[j] The reaction was developed, using p-nitrophenyl phosphate[b] as the substrate, and the OD was read at 405 nm, using a microtitration plate spectrophotometer.[h] Antibody concentrations were calculated with a computer program.[k] The 50% absorbance values of serial dilutions of the serum samples were interpolated onto the linear portion of a standard curve obtained using single-step purified sheep IgG obtained from whole sheep serum by affinity chromatography on protein G-sepharose.[g] Data were expressed as mg of KLH-specific IgG/100 ml of serum; values were transformed to natural logarithms for statistical analysis.

Statistical analyses—On the basis of plasma BHBA concentrations during the last 4 weeks of pregnancy, the ewes were categorized as healthy (BHBA concentration < 0.86 mmol/L) or as having subclinical PT (BHBA concentration > 0.86 mmol/L). Effects of type of feeding (ad libitum vs restricted), type of pregnancy (single vs twin), and BHBA concentration (low vs high) on immune responses were evaluated by use of ANOVA, using the general linear models procedures.[25,l] Interactions between type of feeding, type of pregnancy, and BHBA concentration were not significant and were not included in the final model. Type III sums of squares were used, and data were reported as least-squares means ± SEM. Simple correlation coefficients between BHBA

Table 1—Least-squares mean ± SEM body condition scores (BCS) and plasma glucose, non-esterified fatty acids (NEFA), and β-hydroxybutyrate (BHBA) concentrations in healthy ewes (n = 12) and ewes with subclinical pregnancy toxemia (PT; n = 4)

Weeks before lambing	BCS		Glucose (mmol/L)		NEFA (μmol/L)		BHBA (mmol/L)	
	Healthy ewes	Ewes with PT	Healthy ewes	Ewes with PT	Healthy ewes	Ewes with PT	Healthy ewes	Ewes with PT
4	2.1 ± 0.02	2.0 ± 0.03	3.1 ± 0.11	2.8 ± 0.37	663.1 ± 68.30	1433.3 ± 185.39*	0.32 ± 0.04	0.89 ± 0.04*
3	2.1 ± 0.02	1.9 ± 0.03	3.2 ± 0.14	2.5 ± 0.33*	620.5 ± 56.33	941.8 ± 39.58*	0.38 ± 0.04	1.18 ± 0.06*
2	2.1 ± 0.01	1.9 ± 0.03	3.0 ± 0.12	2.7 ± 0.39	555.3 ± 55.68	802.5 ± 46.28*	0.37 ± 0.04	0.92 ± 0.05*
1	2.1 ± 0.01	1.7 ± 0.02*	3.3 ± 0.13	2.7 ± 0.41*	586.7 ± 58.94	1061.1 ± 34.58*	0.42 ± 0.04	1.30 ± 0.07*

*Significantly ($P < 0.05$) different from value for healthy ewes at the same time period.
Ewes were considered to have subclinical PT if plasma BHBA concentration was > 0.86 mmol/L without clinical signs of disease.

concentrations and immune responses and between NEFA concentrations and immune responses were calculated according to the method of Pearson.[26] For all analyses, values of $P < 0.05$ were considered significant.

Results

None of the ewes developed clinical signs of PT or any other diseases during the study. Amounts of concentrate and forages ingested during the adaptation period were identical for the 2 groups of ewes. Ewes ingested 0.3 kg of the concentrate/head/d (all the amount given) and 1.86 ± 0.43 kg of the forages/head/d (mean ± SEM). During the study, ewes that were fed ad libitum consumed 0.3 kg of the concentrate/head/d (all the amount given) and 1.80 ± 0.62 kg of the forages/head/d. Ewes on restricted feeding did not refuse either concentrate or forages and ingested 0.21 kg of concentrate/head/d and 1.29 kg of forages/head/d. In practice, ewes on restricted feeding ingested 30% less concentrate and 28% less forages than did group-1 ewes.

During the last 4 weeks of pregnancy, 4 ewes (1 group-1 ewe carrying twins and 3 group-2 ewes carrying twins) had plasma BHBA concentrations consistently > 0.86 mmol/L (range, 0.89 to 1.81 mmol/L) and were considered to have subclinical PT. Plasma BHBA concentrations in the remaining 12 ewes were consistently < 0.86 mmol/L (range, 0.31 to 0.48 mmol/L). Three and 1 weeks before expected lambing, mean plasma glucose concentration was significantly higher in healthy ewes than in ewes with subclinical PT (**Table 1**). During the last 4 weeks of pregnancy, plasma NEFA concentration was significantly lower in healthy ewes than in ewes with subclinical PT. One week before expected lambing, mean BCS was significantly lower in ewes with subclinical PT than in healthy ewes.

Type of feeding (ad libitum vs restricted), type of pregnancy (single vs twin), and BHBA concentration (low vs high) were not significantly associated with the increase in double skinfold thickness in response to intradermal injection of PHA. Furthermore, neither type of feeding nor type of pregnancy was significantly associated with in vitro proliferation of PHA-stimulated PBMC or production of KLH-specific IgG. Conversely, in vitro proliferation of PHA-stimulated PBMC and production of KLH-specific IgG were significantly ($P < 0.04$ and $P < 0.004$, respectively) associated with BHBA concentration (< 0.86 mmol/L vs > 0.86 mmol/L). In vitro proliferation of PHA-stimulated PBMC in ewes with subclinical PT (0.73 ± 0.22 OD) was significantly less ($P < 0.05$) than that for healthy ewes (1.4 ± 0.11 OD); whereas, in vitro proliferation of unstimulated PBMC for ewes with subclinical PT (0.04 ± 0.01 OD) was not significantly different from that for healthy ewes (0.04 ± 0.02 OD). Similarly, ewes with subclinical PT had significantly lower values for KLH-specific IgG than did healthy ewes (**Table 2**).

Table 2—Least-squares mean ± SEM concentration of IgG ($\log_e$ of mg IgG/100 ml serum) specific for keyhole limpet hemocyanin in healthy ewes (n = 12) and ewes with subclinical PT (n = 4)

Weeks after initial inoculation*	Healthy ewes	Ewes with PT
1	2.09 ± 0.17	1.44 ± 0.19†
2	2.79 ± 0.12	2.27 ± 0.13‡
3	2.89 ± 0.09	2.59 ± 0.10‡

*Ewes were inoculated intramuscularly with 500 μg of keyhole limpet hemocyanin dissolved in 0.5 ml of sterile phosphate-buffered saline and emulsified in 0.5 ml of incomplete Freund adjuvant (IFA) 4 and 2 weeks before expected lambing. †Significantly ($P < 0.01$) different from value for healthy ewes. ‡Significantly ($P < 0.05$) different from value for healthy ewes.

Finally, plasma BHBA concentration and plasma NEFA concentration were significantly correlated with in vitro proliferation of PHA-stimulated PBMC ($r = -0.78$ [$P < 0.001$] and $r = -0.81$ [$P < 0.002$], respectively), and plasma NEFA concentration was significantly correlated with values for KLH-specific IgG ($r = -0.36$, $P < 0.02$). Plasma glucose concentration was not significantly correlated with in vitro proliferation of PHA-stimulated PBMC or with values for KLH-specific IgG.

Discussion

Results of the present study suggest that subclinical PT in Sardinian sheep adversely affects cell-mediated and humoral immune responses. The fact that all 4 ewes that developed subclinical PT were carrying twins and that 3 of the 4 were on moderate feed restriction testified to the importance of twin pregnancy and undernutrition in the development of this condition.[2,3] Furthermore, results confirmed that high plasma BHBA concentrations are associated with greater decreases in BCS,[a] hypoglycemia, and high plasma NEFA concentrations.[3,7,a]

To our knowledge, studies of the effects of type of pregnancy (single vs twin) or moderate feed restriction on the immune responses of ewes have not been published. Results of the present study indicated that neither type of pregnancy nor moderate feed restriction

themselves significantly altered immune responses of ewes in the late stages of pregnancy.

Results of the present study did indicate that subclinical PT altered cell-mediated immunity of ewes. The lack of correspondence between results of in vivo and in vitro evaluations of cell-mediated immunity may have been a result of the different sensitivities of the 2 assays[27] or, as suggested by others, may indicate effects on cells in vivo that were not present in the in vitro cultures or variations in the populations of lymphocytes (peripheral blood lymphocytes vs lymphocytes in the lymph).[28] Singh et al[14] reported that ewes with clinical signs of experimentally induced PT had a significant depression in cell-mediated immune responses, as assessed by the lymphocyte migration inhibition test and the 2,4-dinitrochlorobenzene skin hypersensitivity test. These authors suggested that the high concentrations of ketone bodies and glucocorticoids that characterize PT in ewes were possible causes for the suppressed cell-mediated immunity.

In a recent in vitro study,[m] we found no effects on proliferation of PBMC cultured with BHBA in concentrations mimicking those in healthy ewes and ewes affected by subclinical or clinical ketosis. Results of studies of the effects of ketosis on cell-mediated immunity in cattle are conflicting. Proliferative responses of mitogen-stimulated PBMC were reduced by incubation of PBMC with supraphysiological concentrations of BHBA[29] or by feeding calves 1,3-butanediol to increase circulating BHBA concentrations.[30] Franklin et al,[31] however, concluded that ketone bodies and glucose at concentrations occurring in vivo had minimal effects on bovine lymphocyte proliferation in vitro. Quigley et al[32] also concluded that increased blood BHBA concentrations in young calves resulting from increased starter intake did not impair immune responses, as evaluated by examining the proliferative responses of mitogen-stimulated PBMC. Finally, in a study with pregnant women, PT was shown to be responsible for a weaker response to intradermal injection of PHA and purified protein-derivative tuberculin.[33]

To date, none of the studies that have found ketosis or high concentrations of ketone bodies in culture medium to have negative effects on cell-mediated immunity have ascertained the mechanisms by which such conditions could impair the function of immune cells. Interestingly, in a recent study on chemotaxis of bovine leukocytes, Suriyasathaporn et al[13] found that leukocytes from ketotic cows or leukocytes cultured in an environment with a high concentration of ketone bodies were less capable of responding to chemotactic stimuli and suggested that differences in the availability of energy in these conditions was a possible cause for the impairment of such a crucial function. The existence of significant relationships between plasma BHBA and NEFA concentrations and measures of cell-mediated immunity and the lack of relationships between plasma glucose concentration and these measures of immunity in the present study would suggest that deficits in glucose played a minor role, compared with the possible toxic effects of high concentrations of BHBA or NEFA.

Results of in vivo and in vitro studies involving other species are conflicting about the effects of various fatty acids on cell-mediated immunity,[34] and information on the effects of NEFA on cell-mediated immunity in ruminants is lacking. However, in a recent in vitro study,[m] we found that various concentrations of NEFA had marked negative effects on proliferation of sheep PBMC. Results of that study and of the present study strongly encourage the assessment of the effects of NEFA on cell-mediated immune response of bovines to explain the immunodepression and related higher incidence of infection in cows with clinical or subclinical ketosis.

Results of the present study indicated that antigen-specific IgG production by ewes in the late stages of pregnancy was negatively affected by subclinical PT. In particular, plasma NEFA concentration was negatively correlated with antibody production. To the best of our knowledge, the relationship between plasma NEFA concentration and antibody production in ruminants has not been studied previously. As for cell-mediated immunity, results of studies involving other species are conflicting about the effects of various fatty acids on antibody production.[34] Nonnecke et al[35] studied the individual and combined effects of ketones, acetate, and glucose on in vitro IgM secretion by bovine blood lymphocytes isolated from healthy Holstein heifers. Conclusions from that study were that in vitro immunoglobulin secretion is not significantly affected by ketones or glucose at concentrations mimicking those associated with ketosis, and additional in vivo studies were necessary. On the other hand, in a study comparing pregnant women with PT and healthy pregnant women, a negative association between PT and total serum IgG and IgA concentrations was observed.[36]

Further studies would be necessary to ascertain whether in vitro concentrations of NEFA and ketone bodies mimicking those associated with subclinical or clinical PT of ewes affect the in vitro production of antibodies by B lymphocytes of sheep. Given the results of the present study, similar studies of the effects of NEFA in cattle would seem warranted. Moreover, as suggested by results of studies by Ford et al[2] and Singh et al,[14] assessment of glucocorticoid concentrations in ewes without clinical signs of disease, but with plasma glucose, NEFA, and BHBA concentrations indicative of subclinical PT, would seem particularly interesting. A possible increase in glucocorticoid concentrations in ewes with subclinical PT may explain,[37] at least partially, the significant decrease in imune response that we observed in the present study. Finally, to determine the biological importance of our findings, further in vivo studies are necessary to establish whether subclinical PT is associated, as in cattle, with an increased susceptibility to other metabolic and infectious diseases of sheep.

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[b]Sigma Chemical Co, St Louis, Mo.

[c]La Veterinaria Strumenti sdf, Padova, Italy.

[d]Mitutoyo Italiana Srl, Lainate (Milano), Italy.

[e]Ficoll-Paque Plus, Amersham Pharmacia Biotech, Milano, Italy.

[f]Corning Glass Works, Corning, NY.

[g]Amersham Pharmacia Biotech, Milano, Italy.

[h]Spectra I, SLT Lab Instruments, Salzburg, Austria.
[i]Nunc, Roskilde, Denmark.
[j]ICN Biomedicals Inc, Costa Mesa, Calif.
[k]Reglin, Fulvio Montauti, Pisa, Italy.
[l]PROC GLM, SAS Institute, Cary, NC.
[m]Lacetera N, Bernabucci U, Ronchi B, et al. Effects of β-hydroxybutyrate, non esterified fatty acids, and urea on Sardinian sheep lymphocytes proliferation (abstr). *J Dairy Sci* 2000;83:43.

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