Effects of Intramuscular Injections of Vitamin B₁₂ on Lactation Performance of Dairy Cows Fed Dietary Supplements of Folic Acid and Rumen-Protected Methionine*

C. L. Girard and J. J. Matte

Agriculture and Agri-Food Canada, Dairy and Swine Research and Development Centre, Lennoxville, QC, Canada J1M 1Z3

ABSTRACT

The experiment was undertaken to determine the effects of i.m. injections of vitamin B₁₂ on lactational performance of primiparous dairy cows fed dietary supplements of folic acid and rumen-protected methionine from 4 to 18 wk of lactation. Fourteen primiparous Holstein cows were assigned to 7 blocks of 2 cows each, according to milk production during the third week of lactation. All cows were fed a basal diet supplemented daily with rumen-protected methionine (18 g of supplement, to bring the estimated supply of methionine to 2.2% of metabolizable protein) plus folic acid (4 mg per kg of BW). Within each block, the cows received a weekly i.m. injection (2 mL) of saline or 10 mg of vitamin B₁₂. Milk production was recorded daily. Milk and blood were sampled every 2 wk. Supplementary vitamin B₁₂ increased energy-corrected milk from 25.8 to 29.0 (SE 1.6) kg/d, as well as milk yields of solids [3.52 to 3.90 (SE 0.22) kg/d], fat [0.87 to 1.01 (SE 0.06) kg/d], and lactose [1.48 to 1.64 (SE 0.11) kg/d]. Supplementation also increased concentrations and amounts of vitamin B₁₂ secreted in milk but had no significant effect on dry matter intake and concentrations and amounts of folates in milk. Packed cell volume, blood hemoglobin, and serum vitamin B₁₂ were increased by supplementary vitamin B₁₂, whereas serum methylmalonic acid was decreased. Serum concentrations of sulfur amino acids were unchanged by treatment. These findings support the hypothesis that, in early lactation, supply of vitamin B₁₂ was not optimal and limited the lactation performance of the cows.

(**Key words:** lactation, vitamin B₁₂, folic acid, rumen-protected methionine)

Abbreviation key: ECM = energy-corrected milk, 5-methyl-THF = 5-methyl-tetrahydrofolate, SAM = S-adenosylmethionine, THF = tetrahydrofolate.

INTRODUCTION

In mammals, 2 enzymes are vitamin B₁₂-dependent. The first, methionine synthase, is essential for transfer of a methyl group from the methylated form of folic acid (5-methyl-THF) to homocysteine for regeneration of methionine and tetrahydrofolate (THF) (Bässler, 1997). The second enzyme is methylmalonyl-CoA mutase, which transforms methylmalonyl-CoA into succinyl-CoA for entry into the Krebs cycle. Methylmalonyl-CoA results from the catabolism of odd-chain fatty acids, and some amino acids (such as valine, isoleucine, methionine, and threonine) and propionate (Le Grusse and Watier, 1993). In dairy cows, this latter reaction is crucial for entry into the Krebs cycle of propionate produced in large amounts in rumen (McDowell, 2000). Optimal lactation responses in dairy cows require key interactions between methionine supply and both vitamin B₁₂ and folate status. In early lactation, serum vitamin B₁₂ is often low, more so in primiparous than multiparous cows (Girard and Matte, 1999). In contrast, supplementation with folic acid increases milk and milk protein yields of multiparous cows but not primiparous cows (Girard and Matte, 1998). In a companion paper (Girard et al., 2005), the interactions between methionine status and folic acid were studied by dietary manipulations. In the current study, methionine and folate status were established at or above requirement in primiparous animals, and the impact of i.m. injections of vitamin B₁₂ on parameters of milk production was monitored during early lactation.

MATERIALS AND METHODS

Cows and Treatments

Fourteen primiparous Holstein cows (BW: 478 ± 10 kg) from the dairy herd at the Agriculture and Agri-Food Canada Research Center (Lennoxville, Canada) were assigned to 7 blocks of 2 cows each according to milk production during the third week of lactation. The cows were kept in a tie-stall barn under 16 h/d of light (0530 to 2130 h) and were milked twice daily at 12-h intervals. Care of cows followed the recommended code

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Corresponding author: Christiane L. Girard; e-mail: girardch@agr.gc.ca.

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of practice of Agriculture Canada (1990) and the guidelines of the Canadian Council of Animal Care (1993). The experiment began at approximately 4 wk of lactation and continued until 18 wk of lactation. All cows were fed a diet described in Girard et al. (2005; companion paper) calculated to supply methionine as 1.75% metabolizable protein, equivalent to 70% of methionine requirement supplemented with rumen-protected methionine (18 g/d, Smartamine M, Rhône-Poulenc Animal Nutrition, Mississauga, ON, Canada). With a methionine content of 88%, 91% rumen by-pass and 97.8% digestibility, the batch of Smartamine M used in the present experiment supplied 14.1 g of methionine per day to bring the estimated methionine supply to 2.2% of metabolizable protein. The cows also received 4 mg of folic acid per kg of BW per d (Rovimix 10% pteroylmonoglutamic acid, Hoffmann-LaRoche, Cambridge, ON, Canada). Those amounts were adjusted for individual BW at the beginning of the experiment. Within each block, the cows received a weekly i.m. injection (2 mL) of either saline (0.9% NaCl; control group) or 10 mg of vitamin B₁₂ (cyanocobalamin, sterile vitamin B12-5000, Rhône-Mérieux, Victoriaville, QC, Canada; B₁₂ group). Every day at 1000 h, orts were removed, and the dietary supplements of folic acid and rumen-protected methionine were incorporated into 500 g of the grass silage used in the total mixed diet. Cows were fed ad libitum. The total mixed diet was distributed in 8 equal meals per day, every 3 h.

Sampling Procedure and Measurements

Milk production was recorded at each milking throughout the experimental period; milk was sampled at 2 consecutive milkings, before the beginning of the experiment (around 3 wk of lactation, d 0) and then, every 2 wk from 8 to 18 wk of lactation. The amount of feed served was recorded daily. Orts were recorded 5 d/wk. For each cow, daily DM intake was calculated per week by subtraction of the average daily refusal from the average intake. Cows were weighed at the beginning of the experiment.

Whole blood and serum were collected by venipuncture of the caudal vein, using a Vacutainer system, at the beginning of the experiment (d 0) and every 2 wk thereafter.

Packed cell volume, hemoglobin, serum concentrations of folates, vitamin B₁₂, homocysteine, methionine, and cysteine, as well as milk composition (total solids, fat, crude protein, ash, and folates) were determined according to the methods described in Girard et al. (2005, companion paper). Vitamin B₁₂ in milk was determined according to the method described by Fie et al. (1994) using a commercial radioassay (Quantaphase

B₁₂, Bio-Rad Laboratories (Canada) Ltd, Mississauga, ON, Canada). Recovery tests were 97.42% and interassay coefficient of variation was 2%.

Serum concentrations of methylmalonic acid were determined using gas chromatography/mass spectrometry according to the modified method of McMurray et al. (1986). Five hundred microliters of blood serum was placed in a 6 × 100 mm borosilicate tube with 20 µL of 30 µM ²H₃-methylmalonic acid (an internal standard), followed by the addition of 500 µL of acetone while vortexing. Then, 1 mL of H₂SO₄ (0.5 M) saturated with NaCl was added, mixed, and followed by 1 mL of ethylacetate. This mixture was vigorously shaken for 25 s and centrifuged at 1200 × *g* for 10 min. The ethylacetate layer was transferred using a disposable Pasteur pipette to a 1-mL glass vial (reacti-Vial). The extraction procedure was repeated 2 times and the 2 extracts were combined. The ethylacetate was removed by evaporation under nitrogen at 20°C using a multineedle manifold (Reacti-Therm III, Heating module, Pierce, Rockford, IL). When the residue was dry, 50 µL of a freshly prepared solution of butylating agent (100 µL of acetylchloride slowly added to 1000 µL of butanol) was added and the samples were vortexed. The tubes were capped and heated at 90°C for 20 min. After the tubes had cooled, 100 µL of hexane was added and mixed, followed by 400 µL of ultrapure water. The layers were allowed to separate and the upper hexane layer was transferred to the gas chromatography vials, which were capped and sealed. An HP6890 gas chromatograph-quadrupole mass spectrometer was used with MS5973 MS. The column was HP-SMS 5% phenyl-methyl siloxane (HP190915). Samples (1 µL) were injected with an autosampler. The capillary injection port was operated in the splitless mode at 280°C. Instrument conditions were as follows: oven injection temperature 65°C increased at 17°C/min to 110°C, then increased at 10°C/min to a final temperature of 250°C. The transfer line and source temperatures were 280 and 250°C, respectively. Measurements were made for *m/z* ions, 119.0 and 122.0.

Statistical Analysis

Energy-corrected milk (ECM) was calculated according to Tyrrell and Reid (1965). At the beginning of the experimental period (approximately 3 wk of lactation), all variables were analyzed using the MIXED procedure of SAS (SAS Institute, 1999) according to a randomized complete block design. When values differed between the treatment groups before the beginning of the experiment (d 0), the variables were analyzed as differences with the values observed at d 0. During the experimental period, all variables were ana-

Figure 1. Serum concentrations of vitamin B₁₂ (least squares means) (SE 22.3) in cows receiving weekly i.m. injections of saline (—□—) or 10 mg of vitamin B₁₂ (—■—) since the fourth week of lactation.

lyzed using the MIXED procedure of SAS (SAS Institute, 1999) according to a randomized complete block design with repeated measures over time. Results are reported as least squares means and SE.

RESULTS

Pre-Experimental Values

The cows started the experiment 21 to 25 d after their first calving. Milk production, ECM, and milk composition were similar for the 2 groups of cows ($P > 0.2$). Blood hemoglobin (10.96 ± 0.32 g/dL), packed cell volume ($29.9 \pm 0.7\%$), serum vitamin B₁₂ (155.2 ± 18.9 pg/mL), methionine (10.02 ± 0.82 μ mol/L), homocysteine (3.23 ± 0.24 μ mol/L), cysteine (192.4 ± 11.4 μ mol/L), and methylmalonic acid (0.593 ± 0.043 μ mol/L) were similar between the 2 groups ($P > 0.2$). Serum folates tended to be lower ($P = 0.07$) in control cows (11.60 ± 2.01 ng/mL) compared with the vitamin B₁₂-supplemented group (14.67 ± 1.46 ng/mL).

Treatment Effects

Blood variables. Serum concentration of vitamin B₁₂ did not change during the experimental period for

control cows but did increase 3-fold with vitamin B₁₂ supplementation (interaction B₁₂ $\times$ time, $P > 0.001$; Figure 1).

Throughout the experimental period, serum folates were higher in cows supplemented with vitamin B₁₂ than in control cows ($P = 0.001$; Table 1). This treatment effect disappeared ($P = 0.40$) when analyzed as differences between values at each sampling time and at d 0 to allow for the differences in pre-experimental values between the 2 groups. Nonetheless, in both groups, serum concentrations of folates during the experimental period were increased by dietary supplements of folic acid; they were 1.5 times those observed before the experiment and did not vary during the experimental period ($P = 0.8$).

Average serum concentrations of methionine, homocysteine, and cysteine were similar between treatments ($P > 0.3$; Table 1) but all altered with stage of lactation (time effect, $P > 0.001$; Table 2).

Throughout the experimental period, supplementary vitamin B₁₂ increased blood hemoglobin ($P = 0.04$) and packed cell volume ($P = 0.06$) but decreased serum concentration of methylmalonic acid ($P = 0.02$; Table 1). Those variables did not differ according to the stage of lactation ($P > 0.3$).

Lactational performance during the experimental period. Supplementary vitamin B₁₂ had no significant effect on milk composition but it increased ECM and yields of milk components (Table 3). Concentrations and amounts of folates secreted in milk were similar for the 2 treatments ($P > 0.4$). The response of those variables to supplementary vitamin B₁₂ was similar throughout the experiment (treatment $\times$ time, $P > 0.3$). Concentrations and amounts of vitamin B₁₂ secreted in milk were increased by injections of vitamin B₁₂ ($P < 0.001$; Table 3). In control cows, the concentrations and amounts of vitamin B₁₂ in milk did not change during the experiment, whereas in cows injected with vitamin B₁₂, the maximum was observed at 10 wk of lactation (treatment $\times$ time, $P < 0.0001$). Changes in milk concentrations of vitamin B₁₂ are illustrated in Figure 2; the

Table 1. Effects of weekly i.m. injections of 0 or 10 mg of vitamin B₁₂ given to primiparous cows from 25 to 125 d of lactation on blood variables (least squares means).

Item	Treatment		SE	P
	Control	B ₁₂		
Serum folates (ng/mL)	17.39	21.68	1.44	0.001
Hemoglobin (g/dL)	11.23	11.88	0.23	0.04
Packed cell volume (%)	31.7	33.2	0.6	0.06
Serum methionine (μ mol/L)	24.73	22.33	1.91	0.27
Serum homocysteine (μ mol/L)	3.50	3.42	0.22	0.54
Serum cysteine (μ mol/L)	223.80	216.10	7.43	0.40
Methylmalonic acid (μ mol/L)	0.586	0.483	0.043	0.02

Table 2 Serum concentrations of sulfur amino acids during lactation (least squares means).¹

	Time after calving (wk)							SE
	6	8	10	12	14	16	18	
Methionine ($\mu\text{mol/L}$)	25.70	20.43	20.60	18.85	21.29	30.46	27.09	2.20
Homocysteine ($\mu\text{mol/L}$)	3.19	3.78	3.09	3.22	3.18	3.77	4.02	0.24
Cysteine ($\mu\text{mol/L}$)	216.64	202.52	180.93	217.56	272.03	236.53	214.47	8.75

¹Time effect, $P < 0.001$.

amounts of vitamin B₁₂ in milk followed an identical pattern (data not shown).

DISCUSSION

In simple terms, folate cofactors are essential to 2 critical functions: biological methylation (via transfer of a methyl group to homocysteine and subsequent production of S-adenosylmethionine, **SAM**) and synthesis of nucleotides (with a key role in formation of purines). These 2 major pathways can compete for folate coenzymes, depending on prevailing physiological demands (Mason, 2003). Tetrahydrofolate obtains 1-carbon units from serine, to form 5,10-methylene-THF, or from formate to form 10-formyl-THF; both are essential for nucleotide synthesis. 5,10-Methylene-THF has an alternative function when, after its irreversible transformation to 5-methyl-THF, it donates a methyl group to homocysteine to yield methionine as an essential part of the methylation cycle (Scott, 1999). This latter reaction involves the B₁₂-dependent methionine synthase and is irreversible. It provides the only route by which THF is regenerated to allow acceptance (and subsequent transfer) of another 1-carbon unit. Therefore, when the

supply in vitamin B₁₂ is low, demethylation of 5-methyl-THF is compromised (Bässler, 1997). This results in folates trapped in a form that cannot be used for DNA biosynthesis (Scott, 1999) and inhibition of the reactions leading to SAM, the major methyl group donor in many metabolite transformations.

From these metabolic considerations, it is clear that the interactions between vitamin B₁₂ and folate status will be complex and influenced by a number of factors, including metabolic demand (e.g., stage of lactation), methionine status, and availability of 1-carbon donors. A number of simple conclusions might be drawn, however. For example, under conditions of low vitamin B₁₂ status, little response would be expected from supply of additional folates, as the regeneration step of 5-methyl-THF to THF would still be compromised. Similarly, with low folate supply, it might be expected that the impact of supplemental vitamin B₁₂ might be marginal, as the rate of THF flux (and consequences for methylation and purine synthesis) may be limited by net supply.

In terms of the impact of supplementary folic acid given to cows with low serum concentrations of vitamin B₁₂, there was no lactational response in primiparous cows (Girard and Matte, 1998, 1999). In contrast, in multiparous cows with serum concentrations of vitamin B₁₂ over 200 pg/mL, additional folates increased milk protein content, milk yield, and milk protein yield (Girard et al., 1995; Girard and Matte, 1998). Similarly,

Table 3. Effects of weekly i.m. injections of 0 or 10 mg of vitamin B₁₂ given to primiparous cows from 25 to 125 d of lactation on lactational performance (least squares means).

Item	Treatment		SE	P
	Control	B ₁₂		
DMI (kg/d)	20.7	21.7	0.8	0.24
Milk production (kg/d)	28.5	31.1	1.6	0.14
Energy-corrected milk (kg/d)	25.8	29.0	1.6	0.03
Solids (g/kg)	122.1	124.8	2.1	0.30
Fat (g/kg)	30.4	32.3	1.2	0.26
Crude protein (g/kg)	33.0	33.2	0.6	0.64
Lactose (g/kg)	51.1	52.4	1.3	0.43
Ash (g/kg)	7.08	7.06	0.08	0.84
Solids (kg/d)	3.52	3.90	0.22	0.01
Fat (kg/d)	0.87	1.01	0.06	0.01
Crude protein (kg/d)	0.95	1.03	0.04	0.20
Lactose (kg/d)	1.48	1.64	0.11	0.01
Ash (kg/d)	0.202	0.221	0.012	0.17
Folates (ng/mL)	58.5	54.6	4.4	0.48
Folates (mg/d)	1.69	1.72	0.2	0.92
Vitamin B ₁₂ (pg/mL)	736	3214	137	0.001
Vitamin B ₁₂ ($\mu\text{g/d}$)	21.1	99.1	3.6	0.001

Figure 2. Milk concentrations of vitamin B₁₂ (least squares means) (SE 188) in cows receiving weekly i.m. injections of saline (—□—) or 10 mg of vitamin B₁₂ (—■—) since the fourth week of lactation.

in multiparous cows fed a diet calculated to meet only 70% of the methionine requirement, but with serum vitamin B₁₂ in excess of 200 pg/mL, supplementary folic acid increased milk total protein and casein contents close to those observed from cows fed rumen-protected methionine (Girard et al., 2005, companion paper). No further improvement was observed when folic acid was administered to cows fed enough rumen-protected methionine to meet requirements, and this suggests that supply of methyl groups could be achieved by additional supply of a key intermediate (methionine) or the vitamin (folic acid) necessary to transfer 1-carbon units from other metabolites (e.g., serine and formate). There was no response to folic acid administration in milk parameters, however, during early lactation when serum vitamin B₁₂ was below 200 pg/mL, but milk parameters did respond to supplemental methionine (Girard et al., 2005, companion paper). This strongly suggests that 1-carbon supply via the methionine cycle, rather than purine biosynthesis, was the rate-limiting step. This is further supported by the present experiment, where dairy cows in early lactation fed a diet supplemented with folic acid and rumen-protected methionine were able to increase milk and milk component yields in response to additional vitamin B₁₂. Although methionine supply and methylation cycle activity might be the more important requirement for these animals, the slight increases in packed cell volume and blood hemoglobin concentrations in cows supplemented with vitamin B₁₂ suggest that, despite an abundant supply of folic acid, DNA biosynthesis was not optimal under the basal conditions.

Although the responses in milk component yields appear to show a clear interaction between vitamin B₁₂ status and folate availability, this would not have been easy to detect from metabolic parameters. For example, the increased production activity might be expected to result with lower concentrations of folates and sulfur amino acids but this was not observed. This may be due to analytical precision or might indicate that serum is not a good index of metabolite status. The metabolic reactions involved, methionine cycle activity and purine biosynthesis, are confined to a limited number of tissues, including the liver and proliferative organs. If these do not show active interchange with the blood then serum would not provide an accurate reflection of metabolic activity. For example, estimates of methionine cycle activity based on plasma isotope kinetics have yielded values in humans (Storch et al., 1988) and sheep (Lobley et al., 1996) much lower than observed within tissues such as liver, kidney, and the small intestine (Lobley et al., 1996).

Low vitamin B₁₂ status can impact the activity of methylmalonyl-CoA mutase and hence utilization of

propionate, with consequences for bioenergetics. In absence of sufficient vitamin B₁₂, methylmalonyl-CoA accumulates and is degraded to methylmalonic acid (Scott, 1999). Serum methylmalonic acid provides a very sensitive index of vitamin B₁₂ deficiency because it is not modified by folate supply (Savage et al., 1994). In the present experiment, the decrease in serum concentrations of methylmalonic acid in cows injected with supplementary vitamin B₁₂ also supports the hypothesis of a suboptimal supply in vitamin B₁₂ in primiparous cows in early lactation. Moreover, some of the effect of supplementary vitamin B₁₂ on milk component yields could have been mediated through an improvement of the efficiency of this metabolic pathway and an increased energy supply.

CONCLUSIONS

In the present experiment, supplementary vitamin B₁₂ increased milk and milk component yields when given in early lactation to primiparous cows fed a diet supplemented with folic acid and rumen-protected methionine. Moreover, the increase in packed cell volume and blood hemoglobin combined with the decrease in serum methylmalonic acid in cows injected with vitamin B₁₂ support the hypothesis that, in early lactation, supply of vitamin B₁₂ was not optimal and limited the lactational performance of dairy cows. These effects may be produced by impacts on use of methionine or propionate, both of which are B₁₂-sensitive.

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