Relationship of Free Fatty Acid and Natural Autoantibodies 2 Weeks Postpartum

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Abstract. Natural autoantibodies (NAAb) have been found in plasma and milk of dairy cows. Natural autoantibodies maintain homeostasis and may prevent infections. The objective of this study was to evaluate the relationship between concentration of plasma free fatty acid (FFA) and the levels of NAAb in plasma binding glutamate dehydrogenase (GD) and carbonic anhydrase (CA) in cows 2 weeks after calving. In total, 55 Holstein-Friesian dairy with 60-d dry period lengths and fed 2 early lactation diets (glucogenic or lipogenic). Blood was sampled at week 1 and 2 after calving. Result showed that from week 1 and 2 relative to calving, levels of IgG and IgM binding CA were positively related with concentration of FFA in plasma. Levels of IgM binding GD were negatively related with concentration of FFA in plasma. It is concluded that IgG and IgM binding CA in plasma might be an indicator for energy status and reflect metabolic status in dairy cows 2 weeks after calving.

1. Introduction

In early lactation, high yielding dairy cows experience a negative energy balance (NEB) caused by a sudden increase of energy requirement for milk production and maintenance while the dietary energy intake in this period is limited. The NEB in early lactation has been related to metabolic disorders[1], infectious diseases[2] and immunosuppression[3]. An elevated level of serum NEFA is one of the indicators of negative energy balance (NEB) in postpartum dairy cows. Previous studies showed that cows with NEB had elevated plasma free fatty acid (FFA) and β-hydroxybutyrate (BHB) concentrations which are related to higher risk of health problems [4-6]. Circulating FFA from lipolysis can be partially oxidized to form ketone bodies or esterified to form triacylglycerol (TAG) in liver because hepatic capacity of complete oxidation is not sufficient[1]. Parts of the formed TAG are exported as very-low-density-lipoproteins (VLDL). It was suggested that FFA and VLDL may be used for milk fat synthesis [7].

Cows with NEB have been attributed to suboptimal immune function like natural antibodies (NAb) in early lactation [3]. In the study of Van Knegsel [12] cows with severe NEB had low NAb. Natural antibodies act as a first line of defence against infection [8, 9] but also to maintain homeostasis, to promote phagocytes of dead cells, and to prevent infectious and autoimmune diseases [10]. Natural antibodies have been divided into two classes: overt and cryptic NAb. Overt NAb bind antigens that the individual has never encountered before. Cryptic NAb or so-called natural autoantibodies (NAAb) are antibodies that bind to self-antigens or slightly changed self-antigens (neo-epitopes). Natural autoantibodies are thought to be involved in inactivation of cytokines, prevention of inflammation, clearance of metabolic waste, perform various homeostatic roles within the immune response [10], and have a role in the prevention of autoimmunity [10].
Earlier study in the present work, cows with higher somatic cell count (SCC) had higher natural antibodies (NAb) titers for isotypes IgG and IgM binding keyhole limpet hemocyanin (KLH) and lipopolysaccharides (LPS) in milk, and higher titer of IgG binding LPS [11]. In an earlier study, high NAb binding myosin in milk of cows were associated with severe NEB ($\beta = -0.001, P < 0.10$), low dry matter intake (DMI) ($\beta = -0.049, P < 0.05$) and occurrence of clinical mastitis [12]. In the study of Van Knegsel [12], NAb binding myosin was measured in milk, but not in plasma. Little is known of the relationship between plasma FFA concentration and NAb in early lactation. In this study, there are two autoantigens will be used glutamate dehydrogenase (GD) and carbonic anhydrase (CA).

2. Material and methods

2.1. Animals and Experimental Design

All the data including animals and experimental design of this study were obtained from the project of WHY DRY at Adaptation Physiology Group Wageningen University, the Netherlands. The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol. Holstein-Friesian dairy cows ($n = 167$) were selected from the Dairy Campus Research dairy herd (WUR Livestock Research, Lelystad, The Netherlands). The experimental design, DP length, and dietary contrasts were described by van Knegsel et al. [13]. In summary, cows were blocked for parity (primiparous or multiparous), expected calving date, milk yield in the previous lactation, and body condition score (BCS). Within blocks, each cow was assigned randomly to a $3 \times 2$ factorial design with 3 DP lengths (0, 30, or 60 d) and 2 early lactation diets (glucogenic or lipogenic). For this study, we used only the data from cows with 60-d DP.

Pre-calving period, lactating cows were fed a lactation diet supporting 25 kg of milk, and dry cows were fed a dry cow ration. From d 10 before the expected calving date and onward, cows of all treatments were fed 1 kg/d of glucogenic or lipogenic concentrate and increased post-calving stepwise with 0.5 kg/d until the concentrate supply reached 8.5 kg/d. The main ingredient for the glucogenic concentrate was corn and the main ingredients for the lipogenic concentrate were sugar beet pulp, palm kernel, and rumen protected palm oil. Forage was supplied ad libitum and was composed of grass silage, corn silage, wheat straw, and rapeseed meal or soybean meal (51:34:2:13, DM basis). Diets were formulated to be isocaloric (Net energy basis; VEM system; [14] and equal in intestinal digestible protein and degraded protein balance (DVE/OEB system; [15]). Blood samples were collected from 56 of the 167 cows, Cows were housed in a free stall with a slatted floor and cubicles and were milked twice daily (0500 and 1630 h).

2.2. Blood Sampling and Analysis

Blood samples were taken from the coccygeal vein at week 1 and 2 after calving at 3 h before the morning feeding. Blood was collected in evacuated tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria) containing EDTA for FFA analysis; or heparin for NAb. Blood samples were centrifuged at 3000 x g at 4°C, for 15 min within two hours after collection. Plasma was transferred and stored at -20°C until analysis.

2.3. Analysis of NAb in plasma of cows

Natural autoantibody titers to two self-antigens GD and CA in plasma of cows were measured by an indirect enzyme-linked immunosorbent assay (ELISA) technique as outlined by Mayasari et al. [24]. In brief, plates were coated with 4 µg/ml of GD (G7882, Sigma) and CA (C3934, Sigma), respectively in 100 µl/well. Natural autoantibodies of the IgG isotype were detected using 1:20,000 (cow) diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish peroxidase (PO) (Cat. No. E10-118P, Bethyl). Natural autoantibodies of the IgM isotype in plasma of cows were detected using 1:20,000 diluted rabbit polyclonal anti-bovine IgM-whole molecule conjugated to PO (Cat. No. A10-
100P, Bethyl). Four step serial dilutions for both IgG and IgM in plasma samples of cows started at 1:40. After washing, a substrate containing tetra methyl benzidine (Sigma Aldrich Chemie, Steinheim, Germany) and 0.05 percent hydrogen peroxide was added, and incubated for 10 minutes at room temperature. The reaction was stopped by adding 1.25 M sulfuric acid. Extinctions were measured with a Multiskan reader (Lab Systems, Helsinki, Finland) at a wavelength of 450 nm. Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50 percent of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) serum present on every microtiter plate[16]

2.4. Statistical analyses
All data of NAAb titers and plasma FFA in cows approximated normality of residuals by examining whether skewness and kurtosis were in a range of -2 until 2. To assess the relationship between NAAb titers in plasma of cows with FFA concentration in plasma of cows, the Pearson Correlation were used. The average, minimum and maximum data from the statistical model and the P-value corresponding to the r are displayed.

3. Results and discussion
As described in the result of plasma FFA concentrations were different over time [29]. The highest level of plasma FFA concentrations of cows with a 60 d dry period (DP) is at week 1 and 2 after calving (Figure 1). Additionally, diet did not affect plasma FFA. Immediately after calving, high milk production coupled with inadequate DMI require the use of energy from body fat stores, which are mobilized as FFA. Most FFA are metabolized in the liver completely into acetyl-coA, incompletely into ketone bodies such as BHBA, or reesterified into triglycerides.

In early lactation, increased mobilization of adipose tissue is associated with high plasma FFA concentration [17]. The increased rate of lipolysis results in increased plasma FFA concentration [18]. Thus, high plasma FFA concentration in cows at week 1 and 2 after calving reflects increased fat mobilization. Free fatty acids can be esterified and stored as TAG in liver. Cows with a moderate (50 to 100 mg/g liver TAG on a wet weight basis) or severe fatty liver (> 100 mg/g liver TAG) were at high risk for metabolic and reproductive problems [18]. Although FFA can be used for ketogenesis through mitochondrial oxidation, we did not found many cows with ketosis in this study. Plasma FFA concentration during early lactation was suggested does not determine the extent of β-oxidation and plasma BHBA concentration [19]. Plasma BHBA could come from the partial β-oxidation of FFA in liver or from conversion of butyrate in rumen [20]. However, plasma BHBA could be used for synthesis of milk fat in the mammary gland [21] or as an alternative energy source for glucose in brain and muscles [22]. Elevated precaval FFA and decreased DMI were associated with impaired neutrophil function [23], which decreases the first line of defence against infectious agents.

In line with previous study that IgM and IgG antibodies binding two self-antigens: GD and CA were detected in plasma of cows [24]. NAAb are present in young and old dairy cows. Previous studies showed that antibodies reacting to self-antigens can be found in plasma and milk from healthy non-immunized dairy cows [12] and in plasma from calves of 20-26 of weeks old [25].

In present study, high plasma FFA concentration was associated with high NAAb both IgG and IgM binding CA at week 1 and 2 after calving. In addition, plasma FFA concentration tended to be associated with IgM binding GD. Previous study showed that cows with subclinical ketosis had higher free fatty acid concentration and GD activation after calving [26]. Carbonic anhydrase also functions in regulation of pH and fluid balance [27] as observed during inflammation [28]. Another study reported NAAb binding myosin and transferrin in milk of cows were associated with sensitivity for mastitis [12]. It is suggested that high plasma FFA concentration was related to regulation of pH and fluid balance during inflammation such as clinical mastitis.
Table 1. Mean, standard deviation, sum, minimum and maximum for the main effects of free fatty acid, natural antibodies (NAAb) isotope IgG and IgM binding glutamate dehydrogenase and carbonic anhydrase.

| Variable                                | N  | Mean | Std Dev | Sum  | Minimum | Maximum |
|-----------------------------------------|----|------|---------|------|---------|---------|
| Non-esterified fatty acid               | 54 | 0.56 | 0.35    | 30.24| 0.07    | 1.94    |
| IgG binding Glutamate dehydrogenase     | 56 | 7.63 | 1.54    | 427.2| 3.8     | 10.8    |
| IgM binding Glutamate dehydrogenase     | 56 | 5.94 | 0.97    | 332.7| 3.8     | 7.5     |
| IgG binding Carbonic anhydrase          | 56 | 5.51 | 1.09    | 308.7| 2.9     | 8.7     |
| IgM binding Carbonic anhydrase          | 56 | 5.48 | 1.02    | 306.6| 3.2     | 9.5     |

Table 2. Pearson Correlation Coefficients between free fatty acid (FFA) and natural antibodies (NAAb) isotope IgG and IgM binding glutamate dehydrogenase dan carbonic anhydrase.

| Natural autoantibodies       | IgG_GD | IgM_GD | IgG_CA | IgM_CA |
|-----------------------------|--------|--------|--------|--------|
| Cows (n)                    | 54     | 54     | 54     | 54     |
| Free Fatty Acid             | 0.17   | -0.25  | 0.52   | 0.24   |
| P-value                     | 0.17   | 0.07   | <0.01  | 0.08   |

n, number of replicates; GD, glutamate dehydrogenase; CA, carbonic anhydrase

Figure 1. Plasma Free fatty Acid (FFA) (mmol/L) at week 1 and 2 after calving. Values represent means (± SEM) per week [29]

4. Conclusion
It is concluded that IgG and IgM binding carbonic anhydrase in plasma might be an indicator for energy status and reflect metabolic status indicated with high plasma free fatty acid concentration in dairy cows 2 weeks after calving. Carbonic anhydrase involved on the regulation of pH and fluid balance during inflammation which occur mostly in early lactation.

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