Blood-derived proteins in milk at start of lactation: Indicators of active or passive transfer

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ABSTRACT

Colostrum has a different composition compared with milk in established lactation. This difference is in part due to the partially open blood-milk barrier, which, when closed, is designed to prevent the interdiffusion of blood and milk components. In the first days of lactation, α -lactalbumin (α -LA), a milk protein, is typically present in blood and several blood-derived proteins are also present in milk, such as IgG₁, IgG₂, serum albumin (SA), and lactate dehydrogenase (LDH). With the exception of IgG₁, which is known to be transferred by active transcellular transport, the other proteins are thought to pass paracellularly through the temporarily open barrier. Along with an exchange of blood and milk components, somatic cell count (SCC) is typically high in colostrum. The decline of these proteins and SCC can be used as indicators to determine transcellular or paracellular transport. Two hypotheses were tested. The first hypothesis was that the decline curve for a protein or SCC would be the same as IgG₁, indicating transcellular transport, or the decline curve would be different than IgG₁, indicating paracellular transport. The second hypothesis was that the decline curves of SCC and all proteins that are thought to have paracellular transport would be the same. Ten Holstein cows were milked at 4 h after parturition, the next 5 consecutive milkings, and the afternoon milking on d 5, 8, 10, and 14 of lactation for a total of 10 milking time points, and sequential jugular blood samples were also taken. Blood and milk samples were analyzed for the concentrations of LDH, SA, IgG₁, IgG₂, and α -LA and milk samples were measured for SCC. Protein concentration and SCC curves were generated from all 10 time points and were evaluated using the tau time constant model to determine the rate of decline of the slope of each protein. When examining the first hypothesis, the concentration of IgG₁ declined significantly faster in the milk than the proteins IgG₂ and LDH, but declined at

the same rate as SA. Immunoglobulin G₁ also declined significantly faster than SCC and α -LA in plasma. The second hypothesis showed that IgG₂, LDH, and SA in milk were declining at the same rate, but were declining significantly faster than SCC and α -LA in plasma. These results indicate that only active transcellular transport of IgG₁ occurred, with a sharp decline at parturition, compared with IgG₂, SA, LDH, α -LA, and SCC, which are likely following paracellular transport. **Key words:** colostrum, blood-milk barrier, blood-derived protein, immunoglobulin, dairy cow

INTRODUCTION

The blood-milk barrier is crucial for the function of the mammary gland to prevent the interdiffusion of blood and milk components, and therefore is highly impermeable during lactation in healthy animals. Most importantly, the blood-milk barrier prevents milk constituents from diffusing into blood, thus allowing the uptake of water from blood plasma into milk driven by an osmotic gradient. Conversely, the barrier prevents blood constituents from being lost by the lactating animal. During colostrogenesis and establishment of lactation, the barrier is not fully functional and can be leaky, allowing for blood components to enter the milk and vice versa (Nguyen and Neville, 1998). Under hormonal regulation of lactogenesis, the blood-milk barrier eventually becomes fully functional and stops the free transfer of blood and milk components (Morgan and Wooding, 1982); however, the precise time when this change occurs is unknown.

Colostrum, or the first milk after parturition, has a different composition than milk in established lactation. Colostrum contains higher concentrations of sodium and chloride, lower concentrations of lactose and potassium, and higher concentrations of immunoglobulins, especially IgG₁ (Nguyen and Neville, 1998; Baumrucker et al., 2010), compared with normal milk. One reason for the differences in colostrum and milk could be the open blood-milk barrier during early lactation, allowing the proteins and ions to pass freely by paracellular means (Linzell and Peaker, 1974). Also in contrast to

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established lactation, the SCC is high during the colostrum period, regardless of the infection status of the mammary gland (Dohoo and Meek, 1982). The SCC can remain elevated for up to 2 wk after parturition (Natske et al., 1972).

Another reason colostrum has a different composition than mature milk is the active transcellular transport of proteins. This is the case for IgG₁, which is the most important immunoglobulin for the passive immunization of the newborn calf through colostrum uptake (Weaver et al., 2000). In cows, IgG₁ is transferred from blood into colostrum via specific transcytosis with the aid of FcRn receptors that are located on the basolateral surface of alveolar mammary epithelial cells (Kemler et al., 1975; Butler, 1983).

Besides IgG₁, several other blood-derived proteins are present in colostrum. These proteins, serum albumin (SA), lactate dehydrogenase (LDH), and IgG₂, are thought to reach the milk through passive paracellular transport as long as the blood-milk barrier is leaky. The function of both SA and LDH in the mammary gland is unclear, but they are indicators of blood-milk barrier permeability (Stelwagen et al., 1994). Immunoglobulin G₂ is an important immune protein but, unlike IgG₁, no specific transfer receptor is known thus far.

During establishment of lactation, the mammary-specific protein α -LA can be found in blood and this is thought to occur by passive paracellular transport (McFadden et al., 1987). α -Lactalbumin is a component of the enzyme lactose synthase that quantitatively determines the biosynthesis of lactose in the mammary gland.

Currently, no studies have investigated the specific decline patterns of proteins and SCC during the first days of lactation compared with the active transfer of IgG₁. The aim of the present study was to examine the decline of proteins in colostrum or plasma during the first days of lactation and use these proteins as indicators of active transcellular transport and passive paracellular transport. The first hypothesis was that the decline curve for a protein or SCC will be the same as IgG₁, indicating transcellular transport, or the decline curve will be different from IgG₁, indicating paracellular transport. The second hypothesis was that the decline curves of SCC and all proteins that are thought to have paracellular transport will be the same.

MATERIALS AND METHODS

Animals

All animal trials were approved and permitted by the Committee of Animal Experiments, Canton of Fribourg, Switzerland (permit no. 2013-01-FR). Ten

Holstein dairy cows [Holstein-Friesian (n = 5), Red Holstein (n = 5)] were selected. Parities of experimental cows ranged from 2 to 5 and no signs of clinical mastitis were noted in any cow during the experimental period. Cows were housed at the Agroscope research station (Posieux, Switzerland) and transferred to straw-bedded calving pens approximately 7 d before parturition. Dry cows were fed roughage ad libitum and 0.5 kg of mineral feed daily until calving. After calving, cows were daily fed roughage, 0.3 kg of mineral feed, 2 kg of protein concentrate, and energy concentrate was fed at 0.5 kg for the first week of lactation and 2 kg the second week of lactation. From d 2 after calving on, cows were allowed to commingle with the herd either in freestall housing or on pasture, and were machine milked regularly (twice daily, 0530 and 1630 h) in the milking parlor between samplings. Cows received 2 oral calcium boluses, the first immediately after calving and the second 4 h after calving. Calves were immediately separated after parturition and were not allowed to nurse their dams.

Experimental Procedures

Beginning 4 h after calving, milk and blood samples were taken. Samples were taken for the first 6 consecutive milkings (morning and afternoon) and during the afternoon milking on d 5, 8, 10, and 14 of lactation. Milking time points were determined according to the milk yield curve published by Kessler et al. (2014). Cows were machine milked completely at every sampling time point and the milk yield was recorded. The milking time was also recorded to determine the time between the sampling and calving. Twenty minutes after the start of milking, cows were restrained and blood samples were taken from the jugular vein with evacuated tubes containing EDTA (Vacuette, Greiner Bio One, Kremsmünster, Austria). Blood was stored on ice until centrifugation at $2,500 \times g$ for 20 min at 4°C to obtain plasma. Milk and plasma samples were stored at -20°C until analysis.

SCC

Milk samples were processed for SCC using a DeLaval cell counter (DCC, DeLaval, Tumba, Sweden) according to the manufacturer's protocol. Samples were diluted 1:10 in commercially available milk if the cell count was $>3 \times 10^6$ because the detection limit for the cell counter is between 3×10^6 and 4×10^6 cells/mL. First colostrum samples were also diluted 1:10 in commercially available milk when viscosity of samples was too high to be read by the cell counter.

SA and α -LA

The concentration of SA and α -LA in milk and plasma samples was analyzed by ELISA using a bovine-specific commercial kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions. Milk samples were diluted 1:12,000 and plasma samples were diluted 1:768,000 for SA and diluted 1:200,000 and 1:60 for α -LA in wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, adjusted to pH 8.0) to ensure the samples were in range of the standards. Absorbance measurements were read on the Synergy Mx plate reader (Biotek Instruments, Winooski, VT). The standard curve for α -LA was 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 ng/mL, and the standard curve for SA was 400, 200, 100, 50, 25, 12.5, and 6.25 ng/mL. The limit of detection was 6.25 ng/mL for SA and 0.78 ng/mL for α -LA. The inter- and intra-assay CV for SA were 4.87 and 4.14% for milk and 4.82 and 4.56% for plasma, 2.95 and 3.08% for milk, and 6.32 and 3.69% for plasma for α -LA, respectively. All analyses were performed in duplicate.

IgG₁ and IgG₂

The concentration of IgG₁ and IgG₂ in milk and plasma samples was analyzed by ELISA using a bovine-specific commercial kit (Bethyl Laboratories) according to the manufacturer's instructions with slight modifications (Lehmann et al., 2013). In short, samples were diluted in wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, adjusted to pH 8.0) to ensure the samples were in range of the standards. The primary and secondary antibodies for IgG₁ were diluted 1:200 and 1:10,000. Primary and secondary antibodies for IgG₂ were diluted 1:200 and 1:15,000. The standard curves were adjusted to 800, 600, 300, 150, 75, 37.5, and 18.75 (ng/mL) for IgG₁ and 500, 400, 200, 100, 50, 25, and 12.5 (ng/mL) for IgG₂. Samples were blocked in blocking buffer containing 5% fish skin gelatin (Sigma-Aldrich, St. Louis, MO) diluted in double-distilled water. Absorbance measurements were read on the Synergy Mx plate reader (BioTek Instruments). The limit of detection was 18.75 ng/mL for IgG₁ and 12.5 ng/mL for IgG₂. The inter- and intra-assay CV for IgG₁ were 3.5 and 13.8% for milk and 5.8% and 9.7% in plasma, respectively. For IgG₂, the inter- and intra-assay CV were 3.6 and 8.6% in milk and 4.3 and 7.2% in plasma, respectively. All samples were performed in duplicate.

LDH Activity

For LDH measurement, milk serum was obtained by a 2-step process (centrifugation at $4,000 \times g$ for

15 min at 4°C, then $14,000 \times g$ for 30 min at 4°C). Lactate dehydrogenase activity was measured in both milk serum and plasma by a commercial kit (Axon-Lab AG, Baden, Switzerland, cross-reacts with bovine LDH) using the COBAS MIRA automated analyzer (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The limit of detection was 0.01 mmol/L.

Modeling and Statistical Analysis

Modeling. To interpret the obtained experimental data, the following model was used. The rate of change for the concentration of each measured protein is assumed to be a linear function of the concentration of the considered protein. More specifically, the decay rate can be written as:

$$\frac{dx(t)}{dt} = -\frac{x(t) - x_s}{\tau},$$

with $x(t)$ the concentration of the considered protein at time t ; x_s the baseline concentration to which the concentration converges; and τ a time constant describing how fast the concentration changes with respect to time. The equation above describes the first-order of linear dynamics. Solving the above differential equation leads to solutions of the following form:

$$x(t) = x_s + (x_o - x_s) \cdot e^{-t/\tau},$$

where x_o is defined as the concentration at time zero and $e^{-t/\tau}$ is defined as an exponential function of time. This function is optimized in least-squares fashion by adjusting the values for x_o , x_s , and τ . Moreover, the following sum of squared residuals (SSR) is minimized:

$$\text{SSR} = \sum_i [y(t_i) - x(t_i)]^2,$$

with $y(t_i)$ the measured concentration at time t_i ; and $x(t_i)$ the corresponding simulated concentration by means of the fitted model. This exercise is executed for each cow and for each measured protein. The τ time constant was calculated for the concentration of IgG₁, IgG₂, LDH, SA, and SCC in milk and α -LA in plasma. The τ time constant value evaluates how quickly a protein declines by 63.2%, and this model also states that $3 \times \tau$ demonstrates how quickly the protein declines to within 95% of its baseline value. All modeling was performed using Matlab modeling and statistical software (Mathworks, Natick, MA).

Statistical Analysis. Data were plotted using Sigmaplot (Systat Software Inc., Chicago, IL) and all statistical analyses were performed using Matlab statistical software (Mathworks). The values for the time constant, τ , obtained for each cow and measured protein were analyzed by pair-wise tests. These tests are used to evaluate the hypothesis that the time constant of the protein IgG₁ is not different from the time constant for another protein (e.g., IgG₂, first hypothesis). These tests are also used to evaluate the hypothesis that the time constant for proteins that are thought to have paracellular transport are not different (second hypothesis). Because substantial variation in the values for these time constants occur within the population of tested specimens, a pair-wise test was considered appropriate. The Wilcoxon signed rank test was used, as an alternative to the Student's *t*-test, because there was neither an equal variance for both of the considered variables or a normal distribution for the sampled values. Values were considered significant when $P < 0.05$. All results are expressed as means $\pm$ SEM.

RESULTS

Ten multiparous cows in their second to fifth lactation (mean lactation = 3.8 ± 0.5) were milked at 10 time points (3.8 ± 0.6 ; 12.4 ± 1.4 ; 25.2 ± 1.6 ; 36.2 ± 1.5 ; 49.1 ± 1.7 ; 60.3 ± 1.6 ; 102.2 ± 3.8 ; 174.2 ± 3.9 ; 222.2 ± 3.8 ; and 318.1 ± 3.8 h) after parturition. Average milk yields of all cows were 8.2 ± 2.1 , 5.5 ± 1.0 , 10.2 ± 1.3 , 11.0 ± 0.8 ; 14.0 ± 1.2 , 13.0 ± 1.0 , $13.0 \pm$

Figure 1. Average milk yield per milking time point, 4 to 324 h after calving. Results are presented as means $\pm$ SEM; $n = 10$ cows.

Table 1. *P*-values for pair-wise tests (protein concentration and SCC)^{1,2}

Parameter	IgG ₁	IgG ₂	SA	LDH	α -LA
IgG ₁					
IgG ₂	0.002				
SA	0.131	0.921			
LDH	0.014	0.160	0.375		
α -LA	0.020	0.049	0.020	0.037	
SCC	0.001	0.001	0.009	0.004	0.193

¹Values determined by the Wilcoxon Sign Rank Test. Values in bold indicate significance ($P < 0.05$).

²SA = serum albumin; LDH = lactate dehydrogenase.

0.9, 14.1 ± 1.1 , 14.7 ± 1.2 , and 14.8 ± 0.7 kg at each milking time point, respectively (Figure 1).

The *P*-values for pair-wise tests for all parameters are presented in Table 1 and τ values are presented in Table 2. For the first hypothesis, IgG₁ ($\tau = 8.0 \pm 1.6$ h, Figure 2A) declined at a significantly faster rate in milk than IgG₂ ($\tau = 11.4 \pm 2.3$ h, Figure 2B) and LDH ($\tau = 13.3 \pm 3.4$ h, Figure 2C), but the decline rate was not statistically different from SA ($\tau = 11.2 \pm 3.3$ h, Figure 2D). Immunoglobulin G₁ also declined significantly faster than α -LA in plasma ($\tau = 46.1 \pm 19.9$ h, Figure 3) and SCC ($\tau = 26.6 \pm 6.1$ h, Figure 4).

For the second hypothesis, no statistical differences were noted between the proteins IgG₂, SA, or LDH in milk, indicating that these proteins were declining at the same rate. Immunoglobulin G₂, SA, and LDH declined at a significantly faster rate in milk than α -LA in plasma and SCC. The rates of decline for SCC and plasma α -LA were not statistically different.

The IgG₁ concentration in milk exceeded the levels in plasma at the first 3 milking time points (4, 12, and 24 h after parturition). The IgG₂, SA, and LDH concentrations in milk did not reach plasma levels throughout the experiment. The IgG₁ plasma concentration (Figure 2A) was increased beginning at 108 h through 324 h after parturition, whereas the plasma levels of IgG₂, LDH, and SA remained relatively constant (Figure 2B, C, D). α -Lactalbumin exhibited a decrease in milk concentration starting at 36 h after parturition (Figure 3).

Table 2. τ -Values¹ for proteins and SCC²

Item	IgG ₁	IgG ₂	SA	LDH	α -LA	SCC
τ	8.0	11.4	11.2	13.3	46.1	26.6
$3 \times \tau^3$	24.0	34.2	33.6	39.9	138.3	79.8

¹ τ -value = how quickly a protein declines to within 63.2% of its baseline value.

²SA = serum albumin; LDH = lactate dehydrogenase.

³ $3 \times \tau$ value = how quickly a protein declines to within 95% of its baseline value.

Figure 2. Immunoglobulin G₁ (A), IgG₂ (B), lactate dehydrogenase (LDH; C), and serum albumin (SA; D) concentration 4 to 324 h after calving. Results and τ values are presented as means $\pm$ SEM. Average concentration in milk (●; mg/mL), average concentration in plasma (○; mg/mL); y-axis left scale is concentration in milk, y-axis right scale is concentration in plasma; n = 10 cows. Average τ value indicates the amount of time (h) it takes for the protein to decline 63.2% to its baseline value.

Figure 3. α -Lactalbumin concentration 4 to 324 h after calving. Results and τ values are presented as means $\pm$ SEM. Average concentration in milk (●; mg/mL), average concentration in plasma (○; ng/mL); y-axis left scale is concentration in milk, y-axis right scale is concentration in plasma; n = 10 cows. Average τ value indicates the amount of time (h) it takes for the protein to decline 63.2% to its baseline value.

DISCUSSION

The aim of our study was to examine the decline patterns of proteins in colostrum or plasma during the first days of lactation and use these proteins as indicators of active transcellular transport and passive paracellular transport. During colostrogenesis, the blood-milk barrier is leaky and allows for the passive interdiffusion of blood and milk components, although there is also an active transport for IgG₁ that enables a high concentra-

Figure 4. Somatic cell count presented in log scale, 4 to 324 h after calving. Results and τ values are presented as means $\pm$ SEM; n = 10 cows. Average τ value indicates the amount of time (h) it takes for the protein to decline 63.2% to its baseline value.

tion in colostrum (Larson, 1985; Nguyen and Neville, 1998).

In the present study, we examined the rate decline of each protein using the time constant, τ , which determines how quickly a protein declines to within 63.2% of its baseline value. This model also states that the $3 \times \tau$ value is 95% of the baseline. This is an appropriate test because it allows for the examination of proteins that are thought to pass through the blood-milk barrier by passive paracellular transport (slower decline) in comparison to IgG₁, which has a known active transport mechanism (rapid decline). The τ time constant was calculated for the concentration of IgG₁, IgG₂, LDH, SA, and SCC in milk and α -LA in plasma. To our knowledge, no other study has used this model and analysis to determine how quickly a protein is declining in concentration in the milk or the blood during the first few days of lactation.

Immunoglobulin G₁ is thought to decline at a faster rate due to the rapid transcellular transport of the protein until parturition with the calving event abruptly ending this transfer (Gross et al., 2014), in comparison to other proteins that are passively diffusing through the open barrier. Using the τ time constant to examine the blood-derived proteins, it was shown that IgG₁ does in fact decline at a faster rate in the milk than IgG₂ and LDH. Interestingly, the decline rate of IgG₁ was not significantly different from SA, although this analysis also showed that the decline rates of SA, IgG₂, and LDH were also not different.

Immunoglobulin G₁ was declining faster in the milk than IgG₂, likely demonstrating that IgG₂ is transferred into milk via a paracellular pathway and the decline is due to the closure of the blood-milk barrier. This difference in decline pattern could also reflect results that the Fc receptor (and active transport via this system) is highly specific to IgG₁ (Larson et al., 1980) compared with other immunoglobulins and that there is still no known transporter for IgG₂.

Remarkably, the τ values indicated that the decline for IgG₁ and SA were not statistically different. Although no known transporter for SA exists, it has been previously shown that SA can noncompetitively bind to the IgG₁ Fc receptor (Chaudhury et al., 2003; Kuo et al., 2010). Chaudhury et al. (2006) demonstrated that the Fc receptor will bind both IgG and SA at different sites with high affinity under low pH conditions, protecting both proteins from degradation. The present results show that it could be possible that a small amount of SA could be transferred across the blood-milk barrier, as the τ values for IgG₁ and SA were not statistically different. In turn, because the τ values for SA were also not statistically different from IgG₂ and LDH, the results demonstrate it is likely not a

significant method of transfer compared with the highly efficient transport of IgG₁. Furthermore, Shamay et al. (2005) showed that explants of mammary tissue could synthesize SA and secrete the protein into the media, indicating that local production exists in the mammary gland. This production could also affect the decline curve and could be an additional interpretation to the fact that there were no statistical differences between the decline of IgG₁ and SA.

In milk, LDH also declined slower than IgG₁, following the same decline pattern as IgG₂, SA, and the closure of the blood-milk barrier. Lactate dehydrogenase, which drives the interconversion of lactate to pyruvate, is a ubiquitous enzyme in the cow and present in all cells. Evidence exists that some LDH is released from damaged cells in the mammary gland (Bogin et al., 1977), but LDH seems to be mainly transferred from the blood into milk (Symons and Wright, 1974; Lehmann et al., 2013). During early lactation increased turnover of cells occurs and there is evidence of a higher incidence of apoptotic cells (Capuco et al., 2001). It is possible that this could contribute to a small release of LDH. The present results show that the amount of LDH released from damaged or apoptotic cells is insignificant, as this protein is following the same decline rate as other blood-derived proteins. Correspondingly, LDH levels in the milk did not reach plasma levels in the current study.

From a biological standpoint, it is important to look at the $3 \times \tau$ value (Table 2), which determines that the protein has decreased to within 95% of its baseline value. For IgG₁, this value at 24 h indicates an abrupt cessation of the active transport of the protein into the milk at calving compared with other blood-derived proteins, which cease transport between 33.6 and 39.9 h. These values also show that the closure of the blood-milk barrier is also limiting the paracellular transport of larger proteins shortly after calving.

α -Lactalbumin is an important component of milk that constitutes around 2.5% of the total milk protein (Davies and Law, 1980). During establishment of lactation, this protein can be found in plasma, which allows it to be an indicator of blood-milk barrier permeability. The levels of α -LA in blood increase and reach a peak at parturition, and decrease shortly thereafter (McFadden et al., 1987). Using the τ time constant, the transport pattern of this protein from milk to blood can be compared with the blood-derived proteins. When α -LA was compared with IgG₁, IgG₂, LDH, and SA, the τ value indicated that this protein decreased at a significantly slower rate. It was expected that α -LA would decrease slower than IgG₁, but decrease at the same rate as IgG₂, LDH, and SA due to the hypothesis that each of these proteins were displaying a cross-wise

(using the same paracellular pathway to diffuse across the blood-milk barrier) passive transport pattern. This discrepancy could be due to differences in the molecular weight of the proteins. The α -LA protein is only 14 kDa, compared with 150 kDa for IgG₂, 140 kDa for LDH, and 66.5 kDa for SA. It is possible that α -LA can continue to pass paracellularly through the blood-milk barrier for a longer period during closure compared with other proteins. α -Lactalbumin concentration also declines in colostrum 36 h after parturition; this finding is in accordance with Levieux and Ollier (1999), who reported similar results.

Importantly, α -LA was still detectable in blood (~200 ng/mL) at 14 d postpartum. Similar results were reported by Sordillo and Akers (1989) that examined the pathology of *Staphylococcus aureus* mastitis during lactogenesis. Their study showed that in noninfected cows the level of α -LA in sera was around 250 ng/mL at 7 d postcalving and close to 200 ng/mL at 14 d postcalving. In the current study, it was also shown that the rate of decline for SCC and plasma α -LA were not significantly different, paralleling the results from McFadden et al. (1987), who described a correlation between milk SCC and plasma α -LA concentrations. Migrating immune cells are able to push epithelial cells off the basement membrane into the lumen (Akers and Nickerson, 2011), and because of these results it was hypothesized that the prolonged presence of α -LA in the plasma was due to its passing through gaps in the epithelium created by sloughed epithelial cells. Interestingly, the decline of IgG₂, SA, and LDH coincided with the closure of the blood-milk barrier, demonstrating that, although there could be gaps in the epithelial cell lining, larger proteins did not pass through the blood-milk barrier after it had begun to close. This may be due to immune cells filling these gaps after they have pushed the epithelial cells into the lumen (Akers and Nickerson, 2011), still not allowing these larger proteins to pass through.

Although the blood-milk barrier is fully functional shortly after calving (as seen by increased milk production and decrease of other blood-derived proteins), it can be speculated that α -LA is detectable at low levels in blood throughout lactation due to cell turnover and also physiological states, such as milk stasis or estrus. A study designed to test α -LA in blood throughout lactation in healthy animals would need to be performed to confirm this hypothesis.

Milking time points (e.g., first 6 consecutive milkings and on 5, 8, 10 and 14 d of lactation) were determined according to Kessler et al. (2014). Their study showed a sharp increase in milk yield between d 2 and 5 and until around d 8 after parturition, where the lactation curve began to flatten and continue more linearly until the

Table 3. Mass values for proteins (mg/milking)^{1,2}

Time point (h)	IgG ₁	IgG ₂	SA	LDH
4	346,677.7	25,774.5	28,225.5	6,175.2
12	79,059.2	7,631.1	8,824.6	2,702.0
24	36,973.7	5,402.5	10,453.5	2,646.3
36	16,231.4	2,850.9	5,813.6	2,283.7
48	13,560.4	2,248.5	5,340.1	2,062.4
60	7,620.3	1,693.3	4,709.9	1,969.3
108	5,299.3	1,002.5	3,161.0	2,613.7
180	3,629.6	746.6	3,829.5	2,270.1
228	2,982.0	702.9	5,578.9	1,808.6
324	2,734.2	729.8	7,358.9	1,315.2

¹Values calculated by multiplying protein concentration by milk yield.

²SA = serum albumin; LDH = lactate dehydrogenase.

peak of lactation. The development of the milk yield in the current paper mirrored that of the previous study performed during the colostrum period by Kessler et al. (2014). It is thought that this sharp increase in milk production is due to the closure of the blood-milk barrier, allowing for water to be pulled into the milk by an osmotic gradient. When junctional complexes in the barrier are formed, a transepithelial electrical potential of 30 to 35 mV exists between the apical and basolateral sides of the epithelium, which is essential for milk secretion (Stelwagen and Singh, 2014). Accordingly, at the time at which a sharp increase in milk yield occurs, the measured proteins are decreasing and reaching a baseline value. With this fact, the mass of each protein (Table 3) was calculated according to milk yield and it was determined that the mass of each protein decreases with time. These results rule out a dilution effect on the proteins by the increasing amount of milk production.

Our study is also unique in that the proteins in question were measured both in the milk and in the blood plasma, as samples were taken sequentially at each milking. This allowed us to examine the concentrations of each protein at the same time points on different sides of the blood-milk barrier. Due to these sequential measurements, it was observed that IgG₁ is the only blood-derived protein that far exceeds plasma levels in milk. Immunoglobulin G₁ levels in milk remained higher than in plasma for the first 3 time points, whereas other blood-derived proteins did not reach plasma levels throughout the experiment. Sordillo et al. (1987) and Larson (1992) showed that, during the colostrum period, IgG₁ in milk is concentrated 5 to 10 times the levels in plasma, although the circulating plasma levels of IgG₁ and IgG₂ are similar. This concentration effect is not seen with IgG₂ (Butler, 1974; Larson et al., 1980). In turn, IgG₁ was also the only blood-derived protein to exhibit a rebound increase in the plasma, beginning an increase at d 5 postpartum. Also, using the data from the sequential milk and blood samplings, we

can look at protein concentrations as a ratio between milk and blood. Using this ratio, we can see that IgG₁ is the only protein that had more than 100% of the protein transferred into the colostrum compared with other proteins. These ratios could also be examined as protein concentration curves and their decline could be measured by the τ time constant. Comparisons in the τ results using these ratios were not different from the concentration results; for example, the IgG₁ ratio curve declined faster than IgG₂ and LDH, but not SA, and there were no statistical differences in the curves for IgG₂, SA, and LDH (data not shown). This is again indicative of a highly efficient transport process for IgG₁ compared with IgG₂, SA, and LDH, which are likely passively entering the colostrum.

The SCC decline in colostrum in comparison to the decline of the aforementioned proteins using the τ time constant was also examined. It was determined that the SCC declined significantly slower than IgG₁, IgG₂, SA, and LDH, but at the same rate as α -LA. The decline of SCC began 4 h after parturition, following the closure of the blood-milk barrier, but remained relatively high throughout the 14 d experiment. On d 14 the average SCC was ~245,000 cells/mL. These results are in agreement with Natske et al. (1972), who found that SCC can remain high for at least 2 wk after calving.

Besides the leaky blood-milk barrier, the reason for high SCC in early lactation is relatively unknown. In humans, it has been proposed that leukocytes, such as neutrophils, can cross the barrier using both transcellular transport and paracellular transport (McDonald and Kubes, 2011), and this is likely occurring in the bovine mammary gland. It is possible that higher production of immune factors at parturition can attract somatic cells into the milk. This can occur to protect the udder under the stress of calving and lactogenesis and is possibly due to the high turnover of cells during this period. Studies examining immune factors in milk would need to be performed to support this idea.

It is important to note that measured somatic cells are composed of immune cells (macrophages, PMNL, and lymphocytes) and also epithelial cells. It is possible for invading immune cells to push epithelial cells off the basement membrane to enter the milk (Akers and Nickerson, 2011). This shows that, along with measuring the migration of immune cells, sloughed epithelial cells already located in the mammary gland can also be measured.

Although elevated SCC does not equal a mastitis infection during the colostrum period, it is possible that undetected subclinical mammary infections were present, as no bacteriology samples were taken. Whereas no cows showed signs of mastitis during our study (e.g.,

blood in milk or clotting), any undetected infections could also influence and increase the SCC.

CONCLUSIONS

Our study showed that, in milk, IgG₁ declined at a faster rate than SCC and the proteins IgG₂ and LDH, but not SA. Immunoglobulin G₁ in milk also declined faster than ALA in plasma. These results, as well as the fact that IgG₂, LDH, and SA declined at the same rate, indicated that IgG₁ follows active transcellular transport, whereas IgG₂, SA, and LDH likely follow passive paracellular transport and the closure of the blood-milk barrier. Further supporting the hypotheses, IgG₁ was the only blood-derived protein that not only reached but exceeded plasma levels in colostrum. Somatic cell count and α -LA in plasma declined at the same rate, but slower than all other proteins. Somatic cell count remained relatively high during the 2-wk sampling period.

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