LACTOFERRIN AND TRANSFERRIN IN BOVINE MILK IN RELATION TO CERTAIN PHYSIOLOGICAL AND PATHOLOGICAL FACTORS

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Ann. Rech. Vét., 1982, **13** *(4)*, 321-328

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Résumé

LACTOFERRINE ET TRANSFERRINE DANS LE LAIT EN RAPPORT AVEC CERTAINS FACTEURS PHYSIOLOGIQUES ET PATHOLOGIQUES. — Les concentrations moyennes de transferrine et de lactoferrine dans le lactosérum de 376 quartiers non-infectés de 42 vaches Holstein × Frisonne prélevé les 30e, 150e et 270e jours de lactation ont été de 0,030 et 0,080 mg/ml respectivement. La concentration moyenne de transferrine dans le sérum a été de 4,63 mg/ml. Le nombre de lactation et la position des quartiers n'ont pas influencé les valeurs de lactoferrine et de transferrine dans le lait. La concentration de lactoferrine a augmenté significativement ($P<0,01$) dans les quartiers non-infectés, passant de 0,03 à 0,06 et à 0,15 mg/ml lors des trois prélèvements successifs. La concentration de transferrine dans le lactosérum n'a augmenté significativement ($P<0,01$) qu'en fin de lactation, passant de 0,025 (30e et 150e jours de lactation) à 0,035 mg/ml (270e jour de lactation). La concentration de lactoferrine a augmenté significativement ($P<0,01$) dans les quartiers infectés par des pathogènes majeurs (41 échantillons), tandis que les infections dues aux pathogènes mineurs n'ont pas provoqué d'augmentation significative. Les valeurs 0,42 et 0,65 ont été trouvées pour les coefficients de corrélation entre les concentrations de lactoferrine et de transferrine dans le lait et le nombre de cellules somatiques, respectivement.

Penetration into the mammary gland by pathogenic bacteria provokes an inflammatory reaction which manifests itself by numerous modifications in milk, which are not always evident, particularly in the case of subclinical and latent mastitis. The problem of diagnosis of non-clinical mastitis by methods other than bacteriological ones has brought numerous authors to study the variations in the chemical composition of milk. In particular the serum albumin (Giesecke and Viljoen, 1974) as well as other proteins such as α-lactalbumin and β-casein and other elements such as lactose (review by Kitchen, 1981).

However, some of the biochemical modifications to the milk's composition are in direct relation with the defense mechanisms which the udder can mobilize in order to face a bacterial attack. Increased passage of blood proteins

towards the milk and stimulation of the local synthesis of antibacterial factors have been reported (Carroll *et al.*, 1963 ; Harmon *et al.*, 1976 ; Hill *et al.*, 1979). As well as the immunoglobulins and the complement system, two proteins, due to their ability to bind firmly with ferric iron, have an antibacterial action and are present in milk : transferrin which comes from the blood and lactoferrin which is synthetized by the glandular epithelium (Harmon *et al.*, 1976). Their bacteriostatic effect on many bacteria has been well documented (review by Bullen *et al.*, 1978), and in certain conditions LF can exert a bactericidal effect (Arnold *et al.*, 1977). By consequence, the appreciation of their role in the defense of the mammary gland merits consideration.

This study is aimed at investigating variations in the concentration of lactoferrin and transferrin in milk in relation to inflammation of the udder. This assessment firstly requires an evaluation of the normal physiological variations of these values. In this aim,variations in concentration of the two proteins in non-infected glands were investigated and related to certain physiological parameters such as age and stage of lactation. The influence of subclinical mastitis was then studied.

Materials and Methods

Herd and experimental design

Forty-two Holstein and Fresian-Holstein cross-bred cows of our experimental herd were used for the study. Quarter foremilk samples were routinely obtained at three week intervals. Data are from milk and blood samples collected at 30, 150 and 270 days of lactation. Several samples were not available for analysis because of culling, blind quarters or losses.

Processing of milk and blood samples

All samples were collected at the evening milking. Milk samples were prepared by centrifugation of whole milk at 1 500 *g* for 20 min to remove cells and fat. Casein was precipitated by addition of rennet and incubation at 37 °C for one hour. Samples were then centrifuged twice at 2 500 *g* for 30 min.

Blood samples were obtained by tail vein puncture. They were allowed to clot at room temperature and sera were separated by centrifugation at 1 000 *g* for 20 min. Serum and whey samples were stored frozen (– 20 °C) until needed.

Bacteriological analysis

An aliquot (25 µl) of a milk sample was spread with a calibrated loop on esculine blood agar plate. Bacteria were identified after 24 and 48 h incubation at 37 °C (Plommet, 1962).

Quarter infection status was categorized as follows :

— No infection : no bacteria isolated from the quarter
— Minor pathogen infection : *Corynebacterium bovis,* micrococci isolated
— Major pathogen infection : *Staphylococcus aureus,* streptococci, *Corynebacterium pyogenes,* coliform, yeast isolated

Somatic cell counting

The procedure recommended by International Dairy Federation (IDF, 1979) was followed. Counting was performed with a Coulter counter (Model F., Coultronics, Margency, France) calibrated monthly with standard milk samples (from INRA, Poligny, France).

Preparation of lactoferrin

Mammary secretions were collected from a cow dried for a month. Whey was prepared by acid precipitation at pH 4.6 and centrifugation at 25 000 *g* for 40 min. The whey was dialysed against 0.05 M NaCl in 5 mM veronal-HCl (pH 7.4) and applied to a 1.0 × 14 cm column of Heparin-Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.05 M NaCl in the veronal buffer, according to Bläckberg (1980), except that the elution step was modified as follows : the column was washed with 50 ml of the same buffer. The eluted proteins were discarded. The column was then eluted with 0.6 M NaCl in the veronal buffer. The red peak emerging from the column was collected and dialysed against 0.2 M sodium acetate buffer. This material was then applied to a 2.5 × 20 cm column of carboxymethyl cellulose (CM 52, Whatman Ltd., Springfield, England). The column was eluted with a linear gradient consisting of 500 ml of 0.2 M sodium acetate as starting buffer and 500 ml of 0.8 M sodium acetate as the limit solvent. The red peak was collected, concentrated using a Diaflo PM 10 membrane in an Amicon stirred ultrafiltration cell (Amicon, Lexington, Ma, USA), dialysed against 0.01 M ammonium bicarbonate, and freeze dried.

Confirmation that the protein preparation was lactoferrin was shown by immunoelectrophoresis against a rabbit antiserum raised against

bovine lactoferrin kindly supplied by A.W. Hill (ARC Institute for Research on Animal Diseases, Compton, Newbury, England). The preparation (50 mg/ml) gave only one precipiting line in immunoelectrophoresis against rabit anti-bovine dry secretion and no line against rabbit anti-bovine serum.

Preparation of transferrin

One volume of bovine serum was diluted with three volumes of 0.005 M Tris-HCl buffer pH 8.8 and then precipitated with four volumes of rivanol (6.9 diamino-2-ethoxy-acridine lactate, Sigma Chemical Company, St-Louis, Mo, USA) at 0.6 % (w/v) at 4 °C. After elimination of the precipitate by centrifugation at 13 000 *g* for 45 min, NaCl was added to a final concentration of 5 % (w/v) to precipitate rivanol. The mixture was agitated overnight at 4 °C then centrifuged and dialysed against phosphate buffered saline. (The pH was brought to 6.5 by adding 1 M acetate-acetic acid buffer pH 6.3). Immunoglobulins were precipitated with 50 % final saturated ammonium sulphate. After centrifugation the supernatant was dialysed against 0.02 M phosphate buffer pH 7.4 and applied to a column of DEAE cellulose (DE 52, Whatman Ltd, Springfield, England) equilibrated in the same buffer, according to Reiter *et al.* (1975). After washing with the equilibrating buffer, elution was done with 0.06 M phosphate buffer pH 7.0, which resulted in two peaks of which the second contained transferrin. This preparation (50 mg/ml) gave only one line in immunoelectrophoresis against rabbit anti-bovine serum.

Preparation of antisera

Antisera were prepared in rabbits by four weekly intradermal inoculations at multiple sites of antigen (1 mg/ml) emulsified in equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mi, USA) for the first injection and Freund's incomplete adjuvant (Difco) thereafter. 100 µg of lactoferrin and 20 µg of transferrin were used per inoculation. Antisera were collected a week after the last injection. The blood was clotted at room temperature and the sera stored at − 20 °C until required. The resulting antisera were tested for their specificity by gel diffusion against the corresponding antigen and bovine serum or dry secretion.

Rabbit anti-bovine dry secretion was prepared with the same inoculation scheme using 0.5 ml of secretion per dose.

Protein quantitation

Transferrin and lactoferrin concentrations in serum and whey were determined by the radial immunodiffusion procedure of Mancini *et al.* (1965). Every whey sample was tested in duplicate and the mean value was used in determining concentration. When duplicate values differed by more than 15 %, the sample was tested again.

Results

Average concentrations of lactoferrin and transferrin from all of the 376 samples of milk from non-infected quarters were respectively 0.080 mg/ml and 0.030 mg/ml. Average concentration of transferrin in serum (122 samples) was 4.63 mg/ml. This average concentration varied little during lactation (table 1). In fact, individual variations during this time were considerable, often with concentrations doubling, though without any systematic trend, which explains why these fluctuations did not influence the average levels. The extreme values (table 1) illustrate the range of these fluctuations.

1. *Influence of physiological factors*

For the study of these factors only data obtained from non-infected quarters were taken into account.

1.1. Influence of lactation number

Neither for lactoferrin nor for transferrin was any systematic trend drawn from the differences noted between animals of different lactation numbers (table 2). The wide dispersion of results can be seen, particularly for lactoferrin. From the significant differences which were recorded, in particular concerning transferrin, no conclusion could be drawn.

1.2. Influence of location of quarters

This analysis was carried out on 16 cows, of which the four quarters remained sterile throughout lactation. This sterility proved true at each of the bacteriological examinations performed every three weeks. No significant difference concerning concentrations of lactoferrin and transferrin in milk was discovered, as a result of location of quarters (table 3).

1.3. Influence of stage of lactation

Concentrations of transferrin in milk at beginning and middle of lactation were similar, but they were significantly greater ($P<0.01$) at the end of lactation (table 1).

Concentrations of lactoferrin at the beginning (30 days), middle (150 days) and end of lactation (270 days) differed significantly from each other ($P<0.01$; table 1). The general tendancy was an increase in values as lactation proceeded. Dispersion of values also increased in the same way.

Table 1. - Transferrin values did not differ in serum but increased significantly in milk in late lactation. Lactoferrin values increased steadily as lactation progressed

Days after calving	(No of samples)	Means ± SD		Range
Transferrin in serum (mg/ml)				
30	(39)	4.67 ± 0.94	a	2.95 - 6.80
150	(41)	4.51 ± 0.56	b	3.25 - 5.50
270	(42)	4.76 ± 0.72	c	3.30 - 6.25
Transferrin in milk (mg/ml) [1]				
30	(126)	0.025 ± 0.010	D	0.010 - 0.065
150	(125)	0.025 ± 0.020	E	0.010 - 0.125
270	(125)	0.035 ± 0.025	DE	0.010 - 0.135
Lactoferrin in milk (mg/ml) [1]				
30	(126)	0.03 ± 0.03	F	0.02 - 0.21
150	(125)	0.06 ± 0.08	F	0.02 - 0.54
270	(125)	0.15 ± 0.13	F	0.02 - 0.47

Means followed by the same letter differ. Capital letters : $P<0.01$; small letters : $P<0.05$.
1 : Data from 376 uninfected quarters.

Table 2. - No systematic trend in mean transferrin and lactoferrin milk values from uninfected quarters were noted in relation with the lactation number

Lactatibn No	(No of samples)	Means + SD	
Transferrin (mg/ml)			
1	(120)	0.03 ± 0.01	A
2	(95)	0.02 ± 0.01	ABC
3	(67)	0.03 ± 0.02	B
4	(56)	0.04 ± 0.02	ABC
5 + 6 + 7	(38)	0.03 ± 0.01	C
Lactoferrin (mg/ml)			
1	(120)	0.06 ± 0.06	DEe
2	(95)	0.06 ± 0.06	FfH
3	(67)	0.12 ± 0.16	DGH
4	(56)	0.12 ± 0.14	EF
5 + 6 + 7	(38)	0.09 ± 0.11	efG

Means followed by the same letter differ. Capital letters : $P<0.01$; small letters : $P<0.05$.

2. *Influence of infection status*

Average value of transferrin quarters infected by a major pathogen was higher than average values of transferrin in non-infected quarters and in quarters infected by a minor pathogen, the latter two being equal (table 4). However, this difference was not found to be significant. The high standard deviation pointed out the great amplitude of different degrees of inflammatory response in quarters infected by a major pathogen. To substantiate this phenomenon, we analysed the relationship existing between somatic cell-count and transferrin concentration in this category. The level of 0.03 mg/ml was kept as the normal average value of transferrin in milk (table 4). It can be seen in table 5 that 63 % (26/41) of quarters infected by a major pathogen

Table 3. - The location of quarters did not influence the transferrin and lactoferrin values in milk of uninfected cows [1]

Lactation No	(No of samples)	Means ± SD
Transferrin (mg/ml)		
Fore-quarters		
right (RF)	(48)	0.03 ± 0.02
left (LF)	(48)	0.03 ± 0.01
Rear quarters		
right (RR)	(48)	0.03 ± 0.02
left (LR)	(48)	0.03 ± 0.01
Lactoferrin (mg/ml)		
Fore-quarters		
right (RF)	(48)	0.09 ± 0.12
left (LF)	(48)	0.08 ± 0.10
Rear quarters		
right (RR)	(48)	0.07 ± 0.09
left (LR)	(48)	0.07 ± 0.07

1 : Data from 16 cows with their four quarters uninfected throughout lactation. Each cow was sampled at 30, 150 and 250 days of lactation.

Table 4. - Major pathogen infections caused an increase in transferrin and lactoferrin values in milk although only the lactoferrin increase was significant

Quarter infection status	(No of samples)	Means ± SD		Range
Transferrin (mg/ml)				
Uninfected	(376)	0.03 ± 0.02	a	0.01 - 0.13
Minor pathogen	(61)	0.03 ± 0.03	b	0.02 - 0.24
Major pathogen	(41)	0.05 ± 0.08	c	0.02 - 0.42
Lactoferrin (mg/ml)				
Uninfected	(376)	0.08 ± 0.09	dF	0.02 - 0.62
Minor pathogen	(61)	0.11 ± 0.12	eg	0.02 - 0.58
Major pathogen	(41)	0.18 ± 0.16	Fg	0.02 - 0.63

Means followed by the same letter differ : Capital letters : $P<0.01$; small letters : $P<0.05$.

Table 5. - Relation between somatic cell-count and transferrin values in samples from major pathogen infections. More infected quarters were detected with somatic cell-count than with transferrin

No of somatic cells ($\times 10^3$/ml)	Transferrin		
	Means ± SD (mg/ml)	No of samples with	
		<0.03 mg/ml	>0.03 mg/ml
<500	0.020 ± 0.004 AC	13	0
500 to 1 000	0.030 ± 0.012 Ab	7	3
>1 000	0.095 ± 0.110 BC	6	12
Total		26	15

Means followed by the same letter differ. Capital letters : $P<0.01$; small letters : $P<0.05$.

contained less than 0.03 mg/ml of transferrin and that 32 % (13/41) of the mastitis was latent, as according to the definition by IDF (1979). The proportion of quarters having a concentration of transferrin higher than the level of 0.03 mg/ml increased, passing from 0 % to 30 % and to 67 %, with passage to a category of higher cellular level. Increase of average value of transferrin was not pronounced except in quarters having more than 10^6 cells/ml (table 5) and in this category values were very widely spread.

Average values of lactoferrin in infected quarters were higher than those in non-infected quarters. However, this average increase was not large enough to be significant, except for the increase recorded in quarters infected by major pathogens ($P<0.01$; table 4). The difference between quarters infected by a minor pathogen and those infected by a major pathogen was also significant ($P<0.05$). Maximal values found in each category were unexpectedly very close (0.58 to 0.63 mg/ml) (table 4).

3. *Iron-binding protein-somatic cell-count relationship*

Correlation between concentration of lactoferrin and transferrin in milk and somatic cell-count was 0.42 and 0.65 respectively, for the total number of samples ($P<0.001$).

Discussion

To our knowledge there is no recent report concerning transferrin in milk. It is therefore difficult to compare our results with those obtained by other authors. The values of transferrin in blood are similar to those reported by Martinsson and Möllenberg (1973). In milk on the 30th day of lactation, concentrations of transferrin and lactoferrin were approximately the same, but afterwards lactoferrin was more abundant. However, the values of transferrin increased at the end of lactation (270th day). The increase in concentration of serum albumin at the end of lactation was reported by Giesecke and Viljoen (1974). Assuming that the transfer mechanism from blood into milk used by these two proteins is passive, one could expect that a change in the gland's physiology favorising the passage of one protein, would also favorise the passage of the other one. In the same way, if the transfer is passive for transferrin, as it is for serum albumin, then their relations as regards the milk/blood concentration should be very close to one another. With the same samples the relation 1:190 was observed with serum albumin (Poutrel *et al.*, 1982) which is close to the ratio 1:150 recorded in this study for transferrin (0.03:4.63). On the 30th day of lactation, Gaunt *et al.* (1980) found an average for lactoferrin equal to 0.128 mg/ml. On the 20th day Senft *et al.* (1976) found this average to be 0.089 mg/ml. These two values are appreciably higher than the value 0.03 mg/ml on the 30th day of lactation which is the value recorded in our study. The precise information on the infectious status of quarters from which samples were taken is not mentioned in the studies of Senft *et al.* (1976) and Klobasa *et al.* (1977), which could partly explain these differences. However, the dispersion of the

results, as judged by their standard deviations, is extremely important in all reports, which corresponds to what we recorded. The extremes reported by the authors above and by Smith and Schanbacher (1977) are close to those observed by us (table 4).

Gaunt *et al.* (1980) found an increase in values of lactoferrin as animals grew older. However, their analysis was carried out on all quarters, both infected and non-infected, which meant that it was not possible to distinguish influence of age from influence of infection status. In some non-infected quarters we observed an increase from the third lactation onwards, but there was no subsequent increase. This observation is of the same kind as that made by Klobasa *et al.* (1975) and it is in keeping with the weak correlation (0.08) found by Harmon *et al.* (1975) between concentration of lactoferrin and number of lactations.

Quarter location did not significantly modify lactoferrin concentration although values in hind quarters were slightly lower (table 3). Milk production and lactoferrin concentration are negatively correlated : − 0.50 according to Harmon *et al.* (1975), − 0.327 according to Gaunt *et al.* (1980). With hind quarters usually having larger milk yield than fore-quarters, one could indeed expect lactoferrin content to be less.

Our results correlate with previous studies on increase in lactoferrin concentration during lactation (Gaunt *et al.*, 1980 ; Senft *et al.*, 1976). Probably, this increase is related to involution of the mammary gland, and it becomes considerably greater after lactation has finished (Welty *et al.*, 1976).

It has been shown that intensity of inflammatory reaction of the mammary gland controls the amplitude of increase in lactoferrin concentration in mastitis secretions (Harmon *et al.*, 1975). In this way mastitis caused by coliforms, which in general is more severe than mastitis caused by staphylococci and streptococci, is accompanied by the highest increase in lactoferrin concentration, reaching 8 mg/ml (Harmon *et al.*, 1975). Modifications in lactoferrin concentration recorded in our study were a lot more moderate. On average, quarters infected by a minor pathogen gave values little different to those of non-infected quarters — average lactoferrin concentration was slightly higher, but not significantly, and average transferrin concentration was not modified. From this it can be concluded that degree of inflammation caused by this category of infection was very moderate, as on average there was no increase in transfer of the blood protein transferrin.

Secretion from quarters infected by a major pathogen contained on average a higher concentration of each of the two proteins studied. However, degree of inflammation was too low to make the difference significant for transferrin. Despite this, the correlation coefficient between transferrin and somatic cell-count (0.65) was similar to those reported for serum albumin (Harmon *et al.*, 1975 ; Smith *et al.*, 1979 ; Poutrel *et al.*, 1982) which means that a relationship exists between transferrin milk content and inflammation. If the case for lactoferrin is examined more closely it can be seen that the average value for infected quarters : 0.18 mg/ml (table 4) differs only very slightly from the average for non-infected quarters at end of lactation 0.15 mg/ml (table 1). Thus the normal physiological variation was of the same amplitude as the variation due to a pathological cause. This observation and in addition the wide dispersion of results around the averages recorded during this study, shows clearly that the diagnosis of non-clinical infections cannot be determined from content of lactoferrin in milk. Table 5 shows that diagnosis value of transferrin is clearly lower than somatic cell-count, as 12/40 quarters infected by a major pathogen elude the diagnosis with a level of 500 000 cells/ml for 25/40 quarters with a level of 0.03 mg/ml of transferrin.

Accepted for publication, September 10th, 1982.

Acknowledgements

Thanks are due to M. Ducelliez for technical assistance and to Stella Russell for assistance with English.

Summary

Mean transferrin and lactoferrin concentrations in whey samples from 376 uninfected quarters of 42 Holstein × Friesian cows on the 30th, 150th and 270th days of lactation were respectively 0.030 and 0.080 mg/ml. The mean transferrin concentration in serum was 4.63 mg/ml. Lactation number and

location of quarters did not influence milk lactoferrin and transferrin values. Lactoferrin concentration increased significantly ($P<0.01$) in uninfected quarters from 0.03 to 0.06 and to 0.15 mg/ml on the three successive sampling times. Transferrin whey concentration increased significantly ($P<0.01$) only in late lactation, from 0.025 (30th and 150th days of lactation) to 0.035 mg/ml (270th day of lactation). Lactoferrin concentration increased significantly ($P<0.01$) in quarters infected by major pathogens (41 samples) whereas minor pathogen infections (61 samples) caused no significant increase. The correlation coefficients between milk lactoferrin and transferrin concentrations and somatic cell-count were 0.42 and 0.65 respectively.

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