Microparticle-enhanced Nephelometric Immunoassay of Lactoferrin in Human Milk

M.L. Cuillière, P. Montagne,* C. Molé, M.C. Béné, and G. Faure

GRIP, Immunology Laboratory, Faculty of Medicine, Nancy, France

A microparticle-enhanced nephelometric immunoassay was developed for lactoferrin quantitation in human milk. It is based on the nephelometric measurement of the light scattered during the competitive immunoagglutination of a microparticle-lactoferrin conjugate with an antilactoferrin antiserum. This immunoassay is sensitive (detection limit in reaction mixture, 0.2 mg/L) and can be performed in diluted milk (1/3,000 in reaction mixture), excluding any interference or sample pretreatment. It allowed the quantification of lactoferrin on a large range of

concentrations (0.675–21.6 g/L) with accuracy (linear recovery in dilution-overloading assay) and precision (within- and between-run coefficients of variation from 3% to 6%). Changes in the lactoferrin concentration of human milk during lactation were determined in 190 samples. The concentration and ratio of lactoferrin vs. total protein were found to be significantly higher in colostrum (5.9 g/L, 29%) than in transitional milk (2.9 g/L, 22%) or mature milk (2.5 g/L, 24%). *J. Clin. Lab. Anal.* 11:239–243, 1997. © 1997 Wiley-Liss, Inc.

Key words: human lactoferrin; human milk; lactation; immunoassay; immunonephelometry; microparticle

INTRODUCTION

Lactoferrin (LF), a major whey component of human milk, is a glycoprotein (1) with a molecular mass of 79 kDa, constituted by a single polypeptidic chain of 703 aminoacids (2) composed of two homologous domains (3). LF is an iron-transport protein (4) that can bind two ferric ions in association with carbonate or bicarbonate (5, 6), but only a small part of this iron-binding capacity is utilized by human milk LF (7). Several biological functions have been proposed for human milk LF and a receptor for LF found in the intestine of newborns (8, 9): a role in iron absorption (8), as a growth factor (10), or and immunomodulatory factor (11). The high affinity of LF for iron and its iron unsaturation allow for a bacteriostatic effect to be exercised by LF, by competing with siderophilic pathogens and thus impeding their proliferation (12). A bactericidal action for a fragment of bovine LF, formed in the gut and containing no iron, also has been suggested in newborns (13). Because of these biological interests, several methods of quantitation of LF in human milk, stool, and urine of breast-fed infants have been reported such as FPLC (14), PAGE (15), and immunoassays (16–19).

Microparticle-enhanced nephelometric immunoassays have been previously described as sensitive and accurate techniques for the determination of various human serum proteins (20). Used for assaying the main components of bovine milk, these assays appeared as an appropriate method for the evaluation

of the quality of bovine milk from production up to processing in the cheese industry (21). These immunoassays are based on the nephelometric quantification of the inhibition of microparticle-antigen conjugate immunoagglutination by the antigen to be assayed (22, 23). In the present work, we report the development of a microparticle-enhanced nephelometric immunoassay of LF in human milk and its application to the investigation of the quantitative changes in milk LF during lactation.

MATERIALS AND METHODS

Reagents

Chromatographically purified human milk LF (90% in SDS-PAGE) was obtained from Sigma Chemical Co. (St. Louis, MO). The purified immunoglobulin fraction of rabbit anti-LF antiserum (anti-LF As) was provided by Dako (Glostrup, Denmark). As suggested by the manufacturer, all traces of contaminating antibodies were removed by liquid-phase adsorption with human plasma proteins, and the reagent's specificity was ascertained using crossed immunoelectrophoresis, which revealed that the LF precipitation arc

*Correspondence to: P. Montagne, Immunology Laboratory, Faculty of Medicine, BP 184, F-54505 Vandoeuvre les Nancy Cedex, France.

Received 4 November 1996; accepted 26 February 1997

only appears against human milk and that no precipitation is observed against human plasma. Polyfunctional hydrophilic microspheres (MS) of 300 ± 12 nm diameter, synthesized as previously reported (23), and the buffer for nephelometry used for all immunonephelometric assays (0.05 M borate buffer, pH 8 containing 0.1 M NaCl, 1.5 mM Na₂-EDTA, 30 mM NaN₃, 2 g/L Triton X-100 and 30 g/L PEG 6000) were supplied by Sanofi-Diagnostics-Pasteur (Marnes, France).

Human milk samples used for precision, recovery, and investigation of the changes in milk LF concentration during lactation were collected from 29 voluntary mothers at the maternity hospital of Nancy (France) or at home. These 190 samples were colostrum (50 samples), obtained from days 2–5 postpartum, transitional milk (44 samples), from days 6–14 postpartum, and mature milk (96 samples), from days 15–86 postpartum. All milk samples were frozen immediately after collection and stored at -20°C until use. They were thawed at 40°C in a water bath and vigorously homogenized, immediately before their analysis. Total protein concentration in these human milks was determined by Bradford's method (24).

Preparation of Microparticle-Lactoferrin Conjugate

Polyfunctional hydrophilic MS were covalently coated with LF through the formation of imine bonds between the aldehyde groups on MS and the ϵ amino group of LF. Human LF (1.6 g) and MS (10 g) were mixed in 1 L of 0.1 M borate buffer, pH 8 containing 0.3 M NaCl. After a 2-hour incubation at room temperature followed by an 18-hour incubation at 4°C , glycine (0.036 M) was added to the binding mixture to block unreacted aldehyde groups on MS. The mixture was further incubated for 2 hours at room temperature. MS-LF conjugate, unbound LF, and glycine excess were separated by centrifugation ($10,000 \times g$, 4°C , 1 hr) on a discontinuous sucrose gradient (200–800 g/L in 0.1 M borate buffer, pH 8 containing 0.3 M NaCl). MS-LF conjugate was recovered at the interface of the sucrose solutions and stored at 4°C , at 1.4 g/L in the binding buffer supplemented by 2 g/L NaN₃.

Assay of Milk Lactoferrin

Microparticle-enhanced nephelometric immunoassay of milk LF was performed in a one-step reaction: 30 μL of unknown or control milk (300-fold diluted) or six serial dilutions (from 1/50 to 1/1,600) of the solution of purified human LF used as standard (3.6 g/L), MS-LF conjugate (30 μL , 1.4 g/L), anti-LF As (30 μL , 130-fold diluted), and nephelometry buffer (210 μL) were mixed together in a reaction microcuvette (Nephelia® microcuvette, Sanofi-Diagnostics-Pasteur). All predilutions were performed in the nephelometry buffer, with a Hamilton (Bonaduz, Switzerland) dilutor. The scattered light was measured with the Sanofi-Diagnostics-Pasteur nephelometer Nephelia® N600 (25) after incubation for 30 min at room temperature.

Reproducibility of the calibration curves was estimated by measuring light scattering for each dilution of the LF standard in 10 successive assays. The precision of the immunoassay was assessed by measuring LF in human milk samples with low, intermediate, and high concentration, 30 times during the same assay (within-run precision) and in 10 successive assays (between-run precision). Analytical recovery was tested in a dilution-overloading experimentation: the dilution assay was performed on serial dilutions of two milk samples containing 4.7 g/L (1/150–1/1,200 and 1/200–1/1,600) and 5.0 g/L of LF (1/200–1/800); the overloading assay was performed in one milk sample (4.7 g/L of LF), diluted (1/1,600), then overloaded by seven increasing amounts of purified LF (1.0–11.5 g/L). The slopes calculated by linear regression analysis for the dilution and the overloading assays were compared using the Student's *t*-test. For the total recovery, including dilution and overloading assays, the null hypothesis H_0 (intercept = 0 and slope = 1) vs. the alternative hypothesis H_1 (intercept $\neq$ 0 and slope $\neq$ 1) were tested by *F* (Fisher) and *t*-tests, respectively.

RESULTS

Microparticle-Lactoferrin Conjugate

Preliminary studies, performed with various concentrations of LF in the mixture of MS coating, showed that the most immunoreactive MS-LF conjugate was obtained with the conditions given in Materials and Methods. The MS-LF conjugate did not autoagglutinate and remained immunoreactive for at least 3 months when stored at 4°C . It was agglutinated by serial dilutions (1/100–1/12,800) of anti-LF As, and the light scattered by conjugate clusters formed during this immunoagglutination could be quantified by nephelometry.

Agglutination of MS-LF conjugate (140 mg/L) with anti-LF As (1,300-fold diluted) was progressively inhibited by graded concentrations of free LF (0.1–12.8 mg/L in the reaction mixture). A 50% inhibition was observed with 1.7 mg/L LF and a minimal concentration of 0.2 mg/L was detectable in the reaction mixture as yielding an intensity of light-scattered 3 SD lower than the mean value obtained in the absence of LF (0% inhibition).

Assay of Milk Lactoferrin

The inhibition of MS-LF conjugate immunoagglutination by six serial concentrations of free LF from 0.225 to 7.2 mg/L in the reaction mixture was retained for establishing the calibration curve of milk LF assay. A calibration range from 0.675 to 21.6 g/L of LF in whole milk (Fig. 1) was thus obtained when the assay was actually performed, as indicated in Materials and Methods, with milk samples 3,000-fold diluted in the reaction mixture. Reproducibility CVs ($n=10$) of the light scattering measured for each concentration of the LF standard used for calibration ranged from 1.6% to a maximum of 3.5%.

Fig. 1. Calibration curve of the microparticle-enhanced nephelometric immunoassay of LF in human milk. Results are plotted as mean $\pm$ SD obtained in 10 successive assays; ●, light scattering in the absence of LF. Assay procedure is given in the text.

The precision of the milk LF immunoassay was assessed by the CVs obtained in within- and between-run studies (from 3.3% to 5.9%) as shown in Table 1. Analytical recovery (Fig. 2) was linear ($n = 18$, $r = 0.992$, $P < 0.001$) for the range of LF concentrations in human milk tested in dilution-overloading assays (0.8–11.4 g/L). A mean percentage of recovery of 96.8% was obtained. The slopes of the dilution (1.003) and overloading (1.136) curves were not significantly different ($P > 0.05$). The slope (1.097) and the intercept (-0.244 g/L) of the total recovery curve, including dilution and overloading assays, were not significantly different ($P > 0.05$) from 1 and 0, respectively.

Application in Human Milk

Application of the microparticle-enhanced nephelometric immunoassay of LF to 190 human milk samples gave the

TABLE 1. Precision of Milk LF Determination

	Within-run precision ($n = 30$) ^a			Between-run precision ($n = 10$)		
Min (g/L)	6.28	2.88	1.62	6.72	2.82	1.16
Max (g/L)	7.28	3.24	1.94	7.45	3.18	1.40
Mean (g/L)	6.69	3.07	1.82	7.10	3.00	1.29
SD ^b (g/L)	0.25	0.10	0.09	0.25	0.11	0.08
CV ^c (%)	3.8	3.3	4.8	3.5	3.8	5.9

^a n = number of assays.

^bStandard deviation.

^cCoefficient of variation.

Fig. 2. Analytical recovery of milk LF. □, ☆, △ and —, dilution assay; ○ and ·····, overloading assay; —, linear regression for total recovery. Experimental details and linear regression parameters are given in the text.

following global results: minimum = 0.7 g/L, maximum = 12.7 g/L, mean = 3.3 g/L, SD = 1.9 g/L. Changes in milk LF concentration and in the ratio of LF/milk total proteins were observed according to the stage of lactation (Fig. 3). The concentration was significantly higher ($P < 0.001$) in colostrum ($n = 50$, minimum = 2.3 g/L, maximum = 12.7 g/L, mean = 5.9 g/L, SD = 2.1 g/L) than in transitional milk ($n = 44$, mini-

Fig. 3. Changes in the LF concentration of human milk during lactation. Results are plotted as mean $\pm$ SD; □ and —, LF concentration; ○ and —, LF percentage among total proteins.

mum = 0.8 g/L, maximum = 4.8 g/L, mean = 2.9 g/L, SD = 1.2 g/L) and in mature milk (n = 96, minimum = 0.7 g/L, maximum = 7.7 g/L, mean = 2.5 g/L, SD = 1.0 g/L). The LF concentration in mature milk was significantly lower ($P < 0.05$) than in transitional milk. The percentage of LF among total proteins varied similarly from colostrum to mature milk: it was significantly higher ($P < 0.001$) in colostrum (mean = 29.1%, SD = 4.3%) than in transitional milk (mean = 21.6%, SD = 1.7%) and in mature milk (mean = 23.8%, SD = 8.3%). However, this ratio was not significantly different ($P > 0.05$) between transitional and mature milk.

DISCUSSION

Nephelometric measurement of the inhibition of MS-LF conjugate immunoagglutination by free LF allowed the development of a microparticle-enhanced nephelometric immunoassay for LF in human milk. This immunoassay is easy to perform (one-step assay, without washing or phase separation) and rapid (30 min, 240 results per hour), allowing specific LF measurement in many milk samples. The concentration of LF in milk is measured over a large range (0.675–21.6 g/L) with high reproducibility (CVs lower than 6% in within- and between-precision) and accuracy (linear recovery in dilution-overloading assay). The inhibition mode chosen protects against the risk of underestimation by antigen excess, which may be encountered in immunoassays based on a non-competitive antigen-antibody reaction. The sensitivity (detection limit, 0.2 mg/L) allows to use high dilutions of milk samples, cancelling sample blank measurement and such clarifying pretreatment as skimming or casein precipitation.

The concentrations of LF measured by this microparticle-enhanced nephelometric immunoassay in 190 human milk samples, collected from 29 mothers and including colostrum, transitional, and mature milks, were distributed over a large range (0.7–12.7 g/L) and varied depending on the stage of lactation. The concentrations obtained in mature milk (2.5 ± 1.0 g/L) were close to those previously reported (1.3–3.6 g/L) using other analytical methods (14–17). Important changes in the absolute and relative LF concentration of human milk were observed during lactation. As previously reported (15–17), LF concentration strongly decreased from colostrum (5.9 g/L, 29%) to transitional milk (2.9 g/L, 22%), but then was relatively unchanged in mature milk (2.5 g/L, 24%). This sharp drop of LF concentration in the early postpartum period is similar to the abrupt change observed for milk IgA levels (16). Secretory IgA and LF being the two major proteins of human colostrum (26), their parallel decrease appears largely responsible for the postpartum fall of the total protein content of human milk, occurring as the volume of milk produced conversely increases.

On account of its easiness and rapidity, the microparticle-enhanced nephelometric immunoassay proposed here for human milk LF measurement could be an appropriate implement

allowing an exhaustive investigation of the quantitative changes in human milk during lactation and providing a better knowledge of the lactogenic response. Such assays could be important to devise better infant formulas providing nutriment as close as possible as human milk to bottle-fed infants.

ACKNOWLEDGEMENTS

We are grateful to Mrs Sieffert, Maternity Hospital of Nancy, who kindly supplied a good many milk samples assayed in this work. Paul Montagne is research engineer at the Institut National de la Santé et de la Recherche Médicale. Marie-Louise Cuillière is study engineer at the Centre National de la Recherche Scientifique.

REFERENCES

1. Spik G, Strecker G, Fournet B, Bouquelet S, Montreuil J: Primary structure of the glycans from human lactotransferrin. *Eur J Biochem* 121:413–419, 1982.
2. Rey MW, Woloshuk SL, De Boer HA, Pieper FR: Complete nucleotide sequence of human mammary gland lactoferrin. *Nucleic Acids Res* 18:5288, 1990.
3. Anderson BF, Baker HM, Norris GE, Rice DW, Baker EN: Structure of human lactoferrin: Crystallographic structure analysis and refinement at 2.8 Å resolution. *J Mol Biol* 209:711–734, 1989.
4. Johansson BG: Isolation of an iron-containing red protein from human milk. *Acta Chem Scand* 14:510–512, 1960.
5. Masson PL, Heremans JF: Metal-combining properties of human lactoferrin (red milk protein). The involvement of bicarbonate in the reaction. *Eur J Biochem* 6:579–584, 1968.
6. Eaton SS, Dubach J, Eaton GR, Thurman G, Ambruso DR: Electron spin echo envelope modulation evidence for carbonate binding to iron(III) and copper (II) transferrin and lactoferrin. *J Biol Chem* 265:7138–7141, 1990.
7. Fransson GB, Lönnnerdal B: Iron in human milk. *J Pediatr* 96:380–384, 1980.
8. Cox TM, Mazurier J, Spik G, Montreuil J, Peters TJ: Iron binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in human intestine. *Biochim Biophys Acta* 588:120–128, 1979.
9. Kawakami H, Lönnnerdal B: Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membranes. *Am J Physiol* 261:841–846, 1991.
10. Nichols BL, McKee KS, Henry JF, Nichols VN: Human lactoferrin stimulates thymidine incorporation into DNA of rat crypt cells. *Pediatr Res* 21:563–567, 1987.
11. Birgens HS, Hansen NE, Karle H, Kristenson: Receptor binding of lactoferrin by human monocytes. *Br J Haematol* 54:383–391, 1983.
12. Stuart J, Norrel S, Harrington JP: Kinetic effect of human lactoferrin on the growth of *Escherichia coli*. *J Biochem* 16:1043–1047, 1984.
13. Tomita M, Bellamy W, Takase M, Yamauchi K, Wakabayashi H: Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J Dairy Sci* 74:4137–4142, 1991.
14. Ekstrand B, Björck L: Fast protein liquid chromatography of antibacterial components in milk: Lactoperoxidase, lactoferrin and lysozyme. *J Chromatogr* 358:429–433, 1986.
15. Sanchez-Pozo A, Lopez J, Pita ML, Izquierdo A, Guerrero E, Sanchez-Medina F, Martinez Valverde A, Gil A: Changes in the protein fractions of human milk during lactation. *Ann Nutr Metab* 30:15–20, 1986.
16. Goldman AS, Garza C, Johnson CA, Nichols BL, Goldblum RM: Im-

- munologic factors in human milk during the first year of lactation. *J Pediatr* 100:563–567, 1982.
17. Hirai Y, Kawaketa N, Satoh K, Ikeda Y, Hisayasu S, Orimo H, Yoshino Y: Concentrations of lactoferrin and iron in human milk at different stages of lactation. *J Nutr Sci Vitaminol* 36:531–544, 1990.
 18. Davidson LA, Lönnerdal B: Lactoferrin and secretory IgA in the feces of exclusively breast-fed infant. *Am J Clin Nutr* 41:852, 1985.
 19. Broxmeyer HE, Bicknel DC, Gillis S, Harris EL, Pelus LM, Sledge GW: Lactoferrin: affinity purification from human milk and polymorphonuclear neutrophils using monoclonal antibody (II2C) to human lactoferrin, development of an immunoradiometric assay using II2C, and myelopoietic regulation and receptor-binding characteristics. *Blood Cells* 11:429–446, 1986.
 20. Montagne P, Laroche P, Bessou Th, Cuillière ML, Varcin P, Duheille J: Measurement of eleven serum proteins by microparticle-enhanced nephelometric immunoassay. *J Clin Chem Clin Biochem* 30:217–222, 1992.
 21. Montagne P, Cuillière ML, Marchal E, El Bari N, Montagne M, Benali M, Faure G, Duheille J, Humbert G, Linden G, Heurtaux N, Blesche JL, Gosselin D, Desmares A, Delahaye D: Application of microparticle-enhanced nephelometric immunoassay of alpha-, beta- and kappa-caseins to evaluate quality of milk, from the production to the valorization in cheese industry. *Lait* 75:221–237, 1995.
 22. Montagne P, Varcin P, Cuillière ML, Duheille J: Microparticle-enhanced nephelometric immunoassay with microsphere-antigen conjugates. *Bioconjugate Chem* 3:187–193, 1992.
 23. Marchand J, Varcin P, Riochet D, Montagne P, Cuillière ML, Duheille J, Pau B: Synthesis of new hydrophilic microspheres: Optimized carriers for microparticle-enhanced nephelometric immunoassay. *Biopolymers* 32:971–980, 1992.
 24. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976.
 25. Cuillière ML, Montagne P, Bessou Th, El Omari R, Riochet D, Varcin P, Laroche P, Prud'homme Ph, Marchand J, Flecheux O, Pau B, Duheille J: Microparticle-enhanced nephelometric immunoassay (Néphélia®) for Immunoglobulins G, A, and M. *Clin Chem* 37:20–25, 1991.
 26. Kunz C, Lönnerdal B: Re-evaluation of the whey protein/casein ratio of human milk. *Acta Pediatr* 81:107–112, 1992.