Changes in the κ -Casein and β -Casein Concentrations in Human Milk During Lactation

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A microparticle-enhanced nephelometric immunoassay was developed for κ -casein quantification in human milk. Together with a previously reported β -casein comparable immunoassay, it was applied to 862 samples of milk, collected from 82 mothers, to investigate the changes in casein concentrations in human milk during the first twelve weeks of lactation. κ -casein immunoassay is sensitive (detection limit in the reaction mixture, 0.02 mg/L) and can be performed in diluted milk, excluding any interference or sample pretreatment. It allowed the quantitation of κ -casein over a large range of concentrations (0.14–4.56 g/L) with accuracy and precision (coefficients of variation from 3 to 10%).

β - and κ -casein concentrations and percentages among milk total proteins increase between colostrum (2.6 g/L, 14.3% and 1.2 g/L, 6.5%, respectively) and transitional milk (4.4 g/L, 33.2% and 1.3 g/L, 9.5%), decrease at different rates from the third to the eighth week, then remain stable at least up to the end of the third month of lactation (2.7 g/L, 25.3% and 0.9 g/L, 8.5%). The β -casein/ κ -casein ratio is higher in colostrum (0.61) than in transitional and mature milk (0.30) and could be related to a better digestibility of colostrum casein micelles by the neonate during the first days of life. J. Clin. Lab. Anal. 13:213–218, 1999. © 1999 Wiley-Liss, Inc.

Key words: κ -casein; β -casein; human milk; lactation; immunoassay; immunonephelometry; microparticle

INTRODUCTION

Beta-casein (β -CN) is the major casein of human milk (1). The main characteristics and functions of β -CN have been previously described (2). With κ -casein (κ -CN), present in milk at a lower concentration, β -CN forms micelles that provide amino acids, calcium, and phosphorus to the developing infant (3). In addition to its nutritional role, an antimicrobial activity of human κ -CN has been reported: κ -CN and the caseinoglycopeptide issued from its proteolysis trigger the growth of *Bifidobacterium infantis* (4) and *Lactobacillus bifidus* (5), inhibiting the colonization of the gut by *Escherichia coli* and protecting the breast-fed infant against gastrointestinal diseases. More recently an inhibitory effect of κ -CN on the adhesion of *Helicobacter pylori* to human gastric mucous cells has also been described (6).

The amino acid sequence of human κ -CN contains 162 residues and is highly glycosylated (7) for a molecular mass of 37 kDa, of which 19 kDa are carbohydrates (8). The structure of casein micelles in human milk results from the association of phosphorylated β -CN and stabilizing glycosylated κ -CN (9,10), though hydrophobic and electrostatic interactions. As in bovine milk, the size of casein micelles in human milk largely depends on the relative amount of κ -CN (11), but human milk micelles, about 43 nm in diameter (12), are smaller than those in bovine milk,

whose diameters are up to 180 nm (13), likely inducing a better digestibility.

Changes in both β -CN and κ -CN levels in human milk during lactation have not been much described, even if several methods (FPLC, polyacrylamide gel electrophoresis, isoelectric precipitation, or electroimmunodiffusion) have been proposed for the quantitation of these caseins, especially for β -CN (2,14,15). Microparticle-enhanced nephelometric immunoassays have been previously described as sensitive and accurate techniques for the determination of various human serum proteins (16) and of the main components of bovine milk, especially κ -CN (17) and β -CN (18). These immunoassays are based on the nephelometric quantitation of the inhibition of microparticle–antigen conjugate immunoagglutination by the antigen to be assayed (19). In addition to the assay previously reported for β -CN in human milk (2), the present work describes the development of a microparticle-enhanced nephelometric immunoassay of κ -CN and their application to the exhaustive investigation of quantitative changes in hu-

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man milk κ -CN and β -CN during the first twelve weeks of lactation.

MATERIALS AND METHODS

Reagents

Whole casein (CN) from human milk (lyophilized powder containing 85% protein as claimed by the manufacturer) was obtained from Sigma Chemical Co. (St Louis, MO). The level of β -CN (71%) and of κ -CN (13%) in this product was determined (2) using polyacrylamide gel electrophoresis in 4.5 mol/L urea, 0.38 mol/L Tris-HCl buffer, pH 8.9, and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (0.1% SDS, 0.38 mol/L Tris-HCl buffer, pH 8.9). A large pool (1L) of fresh milk samples from the milk bank of the maternity hospital of Nancy (France) was skimmed by centrifugation (4,000g, 20 min), stored in aliquots at -20°C , and used for the calibration of milk β -CN and κ -CN measurement. As described below, the concentration of β -CN and κ -CN (4.6 and 1.1 g/L, respectively) in this mixture was estimated by comparison with commercial human CN.

As previously reported (17,18), antisera (As) were prepared by immunizing rabbits with purified bovine β -CN and κ -CN by intradermal injections of 10 mg of each purified casein with Freund's complete adjuvant for the first injection and incomplete adjuvant for subsequent booster injections. Antisera were collected seven days after each booster injection. Their reactivity and specificity were controlled by conventional immunonephelometry. They were not purified to avoid crossreactivity with the other main proteins of bovine milk (from 0.1 to 4%) and their reactivity against human β -CN and κ -CN was preserved.

Polyfunctional hydrophilic microspheres (MS) 300 ± 12 nm in diameter, synthesized as previously reported (20), and the buffer for nephelometry used for all immunonephelometric assays (0.05 mol/L borate buffer, pH 8 containing 1.5 mmol/L ethylene diamine tetraacetic acid (Na₂-EDTA), 30 mmol/L NaN₃, Triton X-100 (2 g/L) and polyethylene glycol 6000 (30 g/L) were supplied by Sanofi-Diagnostics-Pasteur (Marnes, France).

Human milk samples used for precision and recovery of the κ -CN assay and for investigation of the changes in milk β -CN and κ -CN concentration during lactation were collected from 82 benevolent mothers at the maternity hospital of Nancy (France) or at home. These 862 samples were colostrum (179 samples), obtained from days 2 to 5 postpartum, transitional milk (209 samples), from days 6 to 14 postpartum and mature milk (474 samples), days 15 to 28 (294 samples), days 29 to 42 (94 samples), days 43 to 56 (48 samples), and days 57 to 86 (38 samples). 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 milk samples was deter-

mined according to Bradford (21). The study was undertaken in accordance with the ethical standards of the national committee.

Preparation and Immunonephelometric Study of MS-CN Conjugate

Polyfunctional hydrophilic MS were covalently coated with human whole CN according to the protocol previously reported (2). Briefly, human CN (0.5 g) and MS (10 g) were mixed in 1 L of 0.05 mol/L borate buffer, pH 8, then glycine (0.036 M) was added to the binding mixture after 2 h at room temperature then 18 h at 4°C . After a further incubation for 2 h at room temperature, MS-CN conjugate was recovered, by centrifugation (10,000g, 4°C , 1 h), at the interface of a discontinuous sucrose gradient (200–800 g/L in 0.05 mol/L borate buffer, pH 8) and was stored at -20°C , at 3.3 g/L in sucrose (50 g/L), polyethylene glycol 6000 (50 g/L), and NaN₃ (2 g/L) solution.

Immunonephelometric reactivity of MS-CN conjugate with antbovine β -CN As was previously described (2). Accordingly, the Sanofi-Diagnostics-Pasteur nephelometer Nephelia® N600 was used to measure the light scattered during the reaction of MS-CN conjugate (330 mg/L in the reaction mixture) with serial dilutions (1/100–1/12,800 in the reaction mixture) of antbovine κ -CN As. Inhibition of this reaction by graded concentrations (0.1–5.9 mg/L) of human whole CN and serial dilutions (1/1500–1/96,000) of the skimmed pool of human milk samples was compared.

Assay of Milk β -CN and κ -CN

Microparticle-enhanced nephelometric immunoassays of milk β -CN and κ -CN were performed in a one-step reaction: 30 μL of unknown or control whole milk (600-fold diluted) or six serial dilutions (from 1/150 to 1/4,800) of the skimmed pool of human milk samples used as standard (4.6 and 1.1 g/L of β -CN and κ -CN, respectively), MS-CN conjugate (30 μL , 3.3 g/L), antbovine β -CN or κ -CN As (30 μL , 150-fold and 80-fold diluted, respectively), and nephelometry buffer (210 μL) were mixed together in a reaction microcuvette (Nephelia, Sanofi-Diagnostics-Pasteur). All predilutions were performed in the nephelometry buffer, with a Hamilton (Bonaduz, Swizerland) dilutor. The scattered light was measured with the nephelometer after incubation for 1 h β -CN assay) or 30 min (κ -CN assay) at room temperature.

Evaluation of Milk κ -CN Assay

Repeatability of the calibration curves obtained for the κ -CN assay was estimated by measuring light scattering for each dilution of the standard in 10 successive assays. The precision of the immunoassay was assessed by measuring κ -CN in human milks with low, intermediate, and high concentrations 30 times during the same assay (within-run precision) and in 10 successive assays (between-run precision).

Analytical recovery was tested in an experimentation including both dilution and overloading assays: the dilution assay was performed on 30 μ L of two serial dilutions (1/400–1/1,600 and 1/500–1/2,000) of one milk (2.3 g/L of κ -CN); the overloading assay was performed in one milk (0.28 g/L of κ -CN) overloaded by 6 increasing amounts of human whole CN containing from 0.39 to 3.87 g/L κ -CN. Overloaded samples were assayed as unknown milk samples. The slopes calculated by linear regression analysis for the dilution and the overloading assays were compared using 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

Immunonephelometric Reactivity of MS–CN Conjugate With Anti- κ -CN As

The MS–CN conjugate did not autoagglutinate and remained immunoreactive for at least 4 months when stored at -20°C . It was agglutinated by serial dilutions (1/100–1/3,200) of antbovine κ -CN As, and the light scattered by conjugate clusters formed during this immunoagglutination could be quantified by nephelometry.

Agglutination of MS–CN conjugate (330 mg/L in the reaction mixture) with antbovine κ -CN As (800-fold diluted in the reaction mixture) was progressively inhibited by concentrations of free κ -CN ranging from 0.01 to 0.76 mg/L in the reaction mixture, contained in graded amounts (0.1–5.9 mg/L) of human whole CN or serial dilutions (1/1500–1/96,000) of the skimmed pool of human milk samples. Fifty percent inhibition was observed for 0.25 mg/L of κ -CN and a minimal concentration of 0.02 mg/L κ -CN 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 CN (0% inhibition).

Evaluation of Milk κ -CN Assay

The inhibition of MS–CN conjugate immunoagglutination by 6 serial dilutions of the pool of human milk samples (1/1,500–1/48,000) corresponding to κ -CN concentrations ranging from 0.02 to 0.76 mg/L in the reaction mixture was retained for establishing the calibration curve of human milk κ -CN assay. A calibration range of 0.14 to 4.56 g/L of κ -CN in whole milk (Fig. 1) was thus obtained when the assay was actually performed, as indicated in Materials and Methods, with milk samples 6,000-fold diluted in the reaction mixture (30 μ L of sample 600-fold diluted in 300 μ L of reaction mixture). Repeatability CVs ($n = 10$) of the light scattering measured for each dilution of the pool of human milk samples used for calibration ranged from 1.8 to a maximum of 2.7%.

The precision of the human milk κ -CN immunoassay was

Fig. 1. Calibration curve of the microparticle-enhanced nephelometric immunoassay of κ -CN in human milk. Results are plotted as mean $\pm$ SD obtained in 10 successive assays: ■, light scattering in the absence of κ -CN; ●, light scattering by MS-CN conjugate alone. The assay procedure is described in the text.

assessed by the CVs obtained in within- and between-run studies (from 2.4 to 9.9%) as shown in Table 1. Analytical recovery (Fig. 2) was linear ($n = 12$, $r = 0.996$, $P < 0.001$) for the range of κ -CN concentrations in human milk tested in dilution-overloading assays (0.6 to 4.0 g/L). A mean percentage of recovery of $98.9 \pm 7.9\%$ was obtained. The slopes of the dilution (1.06) and overloading (1.11) curves were not significantly different ($P > 0.05$). The slope (1.06) and the intercept (-0.13 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 of β -CN and κ -CN Assays in Human Milk

Microparticle-enhanced nephelometric immunoassays of β -CN and κ -CN were applied to 862 human milk samples. Changes in milk β -CN and κ -CN concentration and in their

TABLE 1. Precision of Human Milk Kappa-CN Determination

	Within-run precision ($n = 30$)			Between-run precision ($n = 10$)		
Min (g/L)	2.77	1.18	0.35	2.70	1.35	0.38
Max (g/L)	3.07	1.34	0.52	3.07	1.47	0.54
Mean (g/L)	2.97	1.29	0.47	2.87	1.39	0.46
SD (g/L)	0.07	0.04	0.03	0.11	0.05	0.05
CV (%)	2.4%	3.0%	7.2%	3.9%	3.2%	9.9%

n, number of assays; SD, standard deviation; CV, coefficient of variation.

Fig. 2. Analytical recovery of milk κ -CN. $\square$, $\triangle$, and $- - -$, dilution assay; $\circ$ and $\cdots$, overloading assay; $—$, linear regression for total recovery. Experimental details and linear regression parameters are given in the text.

ratio among total proteins were observed according to the stage of lactation (Fig. 3).

Similar patterns were observed for the changes in the β -CN and κ -CN absolute concentrations in human milk during lactation, but κ -CN concentration increased less than did the β -CN concentration from colostrum to transitional milk, and decreased more rapidly in mature milk before it remained stable. The percentage of β -CN and κ -CN among milk total proteins increased similarly from colostrum to transitional milk, then decreased in mature milk, but changes were more remarkable for β -CN than for κ -CN relative concentration.

The κ -CN/ β -CN concentration ratio was calculated (mean $\pm$ SD) for each milk sample. This ratio was colostrum (0.61 ± 0.49), then decreased significantly ($P < 0.001$) in transitional milk (0.32 ± 0.12), and remained stable in all 15–84 mature milk samples ($n = 742$, 0.30 ± 0.15).

DISCUSSION

The feasibility and evaluation of the microparticle-enhanced nephelometric immunoassay used for the quantitation of β -CN in human milk have been previously reported (2). Similarly, nephelometric measurement of the inhibition, by free κ -CN, of the MS-CN conjugate immunoagglutination occurring in the presence of anti- κ -CN As, allowed here the development of a microparticle-enhanced nephelometric immunoassay for κ -CN in human milk.

Human CN bound to MS in MS-CN conjugate and antibovine β -CN and κ -CN As were used in these human CN assays. Alignment of the amino-acid sequences (22) of human and bovine β -CN and κ -CN precursors, performed us-

Fig. 3. Changes in the β -CN and κ -CN levels of human milk during lactation. Results are plotted as mean $\pm$ SD. $\square$, β -CN and $\diamond$, κ -CN: absolute concentration; $\blacksquare$, β -CN and $\blacklozenge$, κ -CN: relative percentage among total proteins; *, concentration or percentage significantly different ($P < 0.05$) between the period investigated and the previous one.

ing Internet-based tools such as Swiss-Prot sequence library (<http://expasy.hcuge.ch/sprot/sprot-search.html>) and appropriate softwares from EERIE (<http://genome.eerie.fr/fasta/align-query.html>) and EMBL (<http://www.embl-heidelberg.de/predictprotein.html>), shows an identity of 57% for β -CN and of 54% for κ -CN in 233 and 182 amino acid overlaps, respectively. For β -CN, the conserved residues are distributed along the entire length of the molecule and may play an important role in providing the same three-dimensional structure and physico-chemical properties between bovine and human β -CN (1). For κ -CN, the region around the peptide bond between Phe97 and Ile98, the proteolytic cleavage of

which leads to the formation of para- κ -CN and caseinoglycopeptide (23), is particularly well conserved (24). These large degrees of homology and the crossreactivity between human and bovine CN (25) allowed us the use of As prepared by immunizing rabbits with bovine β -CN and κ -CN.

The human milk κ -CN immunoassay developed here is easy to perform (one step assay, without washing or phase separation) and rapid (30 min, 240 results per hour), allowing κ -CN measurement in many milk samples. The concentration of κ -CN in human milk is measured over a large range (from 0.14 to 4.56 g/L) with a good reproducibility (CVs from 3 to 10 % 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 noncompetitive antigen-antibody reaction. The sensitivity (detection limit, 0.02 mg/L) allows the use of high dilutions of whole milk samples, cancelling sample blank measurement and such clarifying pretreatment as skimming or casein separation. On account of these characteristics, the microparticle-enhanced nephelometric immunoassay developed here for κ -CN measurement in human milk and that previously reported for β -CN (2) appeared appropriate for an exhaustive investigation of the quantitative changes in these nutritionally available proteins of human milk during lactation.

The concentrations of β -CN and κ -CN measured by these microparticle-enhanced nephelometric immunoassays in 862 human milk samples, collected from 82 mothers and including colostrum, transitional, and mature milks, were distributed over a large range (from 0.25 to 9.10 and 0.14 to 2.93 g/L for β -CN and κ -CN, respectively). Important changes in the absolute and relative β -CN and κ -CN concentrations of human milk were observed depending on the stage of lactation. As previously reported, especially for β -CN and for limited samplings, by some authors using other analytical methods (2,14,15), β -CN and κ -CN absolute concentrations and their relative percentages among total proteins increased from colostrum to transitional milk, then decreased at different rates from transitional to mature milk in which they became stable at least up to the end of the third month of lactation, thus supplying breast-fed infants with food intake for growth and development. Our results establish normal ranges (95% of the population) for β -CN (0.7–4.7 g/L) and for κ -CN (0.2–1.4 g/L) in healthy lactating mothers with well-established lactation.

A decrease of the concentration ratio of κ -CN vs. β -CN was observed from colostrum (0.61) to transitional milk (0.32) and mature milk (0.30). Decrease of the corresponding molar ratio (from about 0.4 in colostrum to 0.2 in transitional and mature milk) could induce an increase in the size of the CN micelles during the same period (10,11). The consistency of the curd, produced in the stomach of the neonate during clotting consecutive to the proteolysis of κ -CN by pepsin with

liberation of caseinoglycopeptide (23), and coalescence of micelles by hydrophobic interactions between para- κ -CN molecules could be thus thinner for colostrum than for mature milk. Further proteolysis in the stomach and duodenum (26) could be consequently easier for colostrum curd than for mature milk curd and the structure of CN micelles in colostrum best adapted to the physiological capabilities of the neonate to digest milk during the first days of life.

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