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C3/C4 Concentration Ratio Reverses Between Colostrum and Mature Milk in Human Lactation

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The levels of complement fractions C3 and C4 were assayed in human milk in a classic nephelometric assay adapted to this secretion. Concentrations of these molecules were measured in 667 milk samples obtained sequentially from 76 volunteer lactating mothers during the first 12 weeks of lactation. Immunonephelometry was performed using skimmed milk samples diluted 10 times and yielded reproducible (coefficients of variation in within- and between-run precision lower than 9% for C3 and than 14% for C4) and accurate (linear recovery in dilution-overloading assay) data. High concentrations (mean ± SE) were found for C3 (199.32 ± 16.35 mg/L) and C4 (113.42 ± 11.16 mg/L) in colostrum samples (n = 159; days 1–5). A significant ($P < 0.001$) and rapid decrease was observed in transitional milk samples (n = 198; days 6–14), containing 57.71 ± 5.18 and 72.39 ± 4.98 mg/L of C3 and C4, respectively. Stable lower levels were noted in mature milk samples (n = 310; days 15–84) at 30.36 ± 1.57 mg/L for C3 ($P < 0.001$) and 53.38 ± 3.61 mg/L for C4 ($P < 0.05$). The decrease rate was different for C3 and C4, yielding a reversal of the C3/C4 ratio between colostrum and more mature milk.

KEY WORDS: Complement components; human milk; immunonephelometry; lactation.

INTRODUCTION

The complement system belongs to innate immunity, as it provides efficient and rapid protection, even in the absence of cognate interactions. This complex and highly regulated system comprises about 30 soluble and membrane glycoproteins (1). Activation of the complement cascade depends on the successive activation of fraction precursors through a series of regulated enzymatic activities. Three activation pathways have been described, the classical, alternate, and lectin pathways (2). C3 and C4 appear to be important complement fractions, owing to their high serum levels, with mean concentrations of 1230 ± 260 mg/L (range, 860–1840 mg/L) for C3 and 436 ± 118 mg/L (240–800 mg/L) for C4 as determined by radial immunodiffusion (RID) (3). Both also have key functions as C3 convertases in the C4b2a and C3bB complexes.

Complement fractions are routinely assayed in serum samples by immunoprecipitation techniques (4), most frequently nephelometric (5, 6), although ELISA assays (7) have also been proposed. Only a few studies have been interested in measuring C3 and C4 concentrations, using preferentially RID, in human milk (8–13) and focused on the influence of specific maternal conditions: nutritional status, local infections (14–16) or influence of parity, and age of the mother (17). Human milk is a rich and complex nutriment aiming at providing both food and protection to the immature newborn. Milk is a sterile medium, and the presence of complement fractions in this secretion is probably of no relevance to the mammary gland. However, as the saprophyte flora develops in the newborn's upper respiratory tract and gut, it may be highly important that milkborne components of innate immunity regulate colonization. Several studies (18–20) have shown that colostrum differs radically from mature milk and that an intermediate secretion is produced before the composition of milk stabilizes. We investigated the participation of the complement fractions C3 and C4 in the composition of human colostrum, transitional and mature milk.

METHODS

Milk Samples

Human milk samples were collected by 76 volunteer lactating women at the local maternity or at home. A

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total of 667 human milk samples was tested including 159 colostrum samples obtained between day 1 and day 5 postpartum, 198 transitional milk samples collected between day 6 and day 14 postpartum, and 310 mature milk samples collected between day 15 and 86 postpartum. All milk samples were aliquoted, then frozen at −20°C just after collection and stored until they were used. The samples were thawed at 40°C in a water bath and vigorously homogenized before the assays. Preclearing was achieved by adding (v/v) 1,1,2-trichlorotrifluoroethan (Merck, Darmstadt, Germany). After thorough mixing and centrifugation for 20 min at 4900*g*, assays were carried out using the recovered upper phase of each sample.

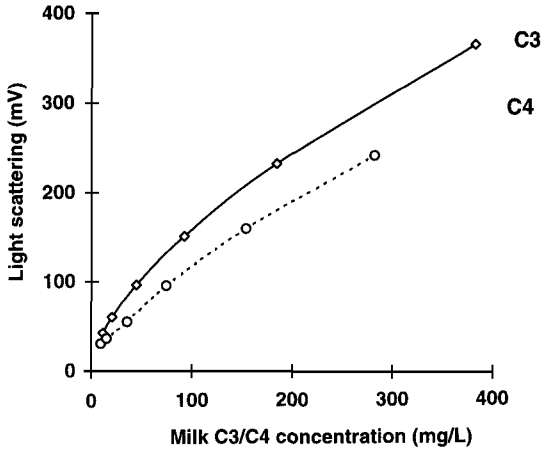

Fig. 1. Calibration curve of the C3 and C4 nephelometric immunoassays in human milk.

Immunonephelometric Assay of Milk C3, and C4 Complement Fractions

Calibration curves were performed with serial dilutions of a calibrator (from 1/20 to 1/640 for C3 and from 1/10 to 1/320 for C4) purchased from Behring Diagnostics (Marburg, Germany). After reconstitution of the lyophilized product in distilled water, this calibrator, based on reference IFCC/BCR/CAP, contained 740 mg/L of C3 and 292 mg/L of C4. All milk and calibrator dilutions were prepared with an automated dilutor (Hamilton, Bonaduz, Switzerland) in 0.01 M phosphate buffer, pH 7.2, containing 0.14 M NaCl.

Both C3 and C4 nephelometric immunoassays were performed in reaction microcuvettes (Nephelia microcuvette; Sanofi-Diagnostics-Pasteur, Marnes la Coquette, France) by mixing 100 μl of skimmed milk samples diluted 10 times or diluted calibrator with 40 μl of anti-human C3/C3c or C4 rabbit antiserum (Behring) and 160 μl of nephelometry buffer [0.05 M borate buffer, pH 8.3, supplemented with 1.5 M Na_2-EDTA, 2 g/L Triton X-100, and 56.25 g/L polyethylene glycol (PEG) 6000]. The scattered light was measured with a Sanofi-Diagnostics-Pasteur-Nephelometer Nephelia N600 after 30 min of incubation at room temperature.

The reproducibility of the calibration curve was provided by measuring, in 10 successive assays, the light scattering of a 40-fold diluted calibrator. The precision of C3 and C4 immunoassays was estimated by measuring, 12 times during the same assay (within-run) and in 10 successive assays (between-run), C3 or C4 concentrations in milk samples with low, intermediate, and high levels. Dilution and overloading experiments were performed to study the analytical recovery. The dilution assay comprised two (1:10–1:160 and 1:16–1:128) and three (1:10–1:40, 1:8–1:32, and 1:12–1:48) serial dilutions of the same milk sample for C3 and C4, respectively. The overloading assay was performed by adding, in increasing levels, a milk sample with a high C3 (381.1 mg/L) or C4 (82.6 mg/L) concentration to a sample with a low C3 (45.2 mg/L) or C4 (16 mg/L) concentration. Linear regression analysis of the dilution and overloading assays allowed us to determine their slopes, which were compared by Student's *t* test. The null hypothesis H0 (slope = 1 and intercept = 0) vs the alternative hypothesis H1 (slope ≠ 1 and intercept ≠ 0) were tested for total recovery, including dilution and overloading assays, by Fisher and *t* tests, respectively.

RESULTS

Calibration ranges from 11.56 to 370 mg/L for C3 and from 9.12 to 292 mg/L for C4 were obtained in human milk when the assays were performed on samples diluted 10 times, as described under Methods (Fig. 1). The reproducibility of the calibration, reflected by the coefficient of variation (CV) of the measurement of the scattering light ($n = 10$) in the 40-fold diluted standard, was 4.7% for C3 and 4.1% for C4.

The precision evaluated by the CVs, determined in within- and between-run assays, with high, medium, and low C3/C4 concentration samples to cover a large range of the calibration curve, was lower than 9% for C3 and less than 14% for C4 immunoassay, as reported in Table I.

The slopes of C3 dilution (1.015) and overloading curves (1.021) were not significantly different ($P > 0.05$), nor were those of C4 dilution (1.053) and overloading (1.013) (Fig. 2). The analytical total recovery (dilution and overloading assays) of C3 appeared to be linear ($n = 16$; $r = 0.998$, $P < 0.001$) for concentrations

Table I. Precision of C3 and C4 Determination in Human Milk with High, Medium, Low Concentrations[a]

	Minimum (mg/L)	Maximum (mg/L)	Mean (mg/L)	SD (mg/L)	CV (%)
Within-run precision[b]					
C3					
High	218.31	252.23	239.25	10.01	4.2
Medium	71.39	76.86	74.72	1.64	2.2
Low	28.15	31.09	29.58	0.85	2.9
C4					
High	165.92	189.79	181.47	6.78	3.7
Medium	74.58	81.04	77.11	1.64	2.1
Low	28.30	31.70	30.00	1.11	3.7
Between-run precision[c]					
C3					
High	186.31	214.55	196.84	9.34	4.7
Medium	42.07	50.13	46.14	2.66	5.8
Low	21.61	27.54	24.29	2.11	8.7
C4					
High	150.12	169.19	160.46	6.47	4.0
Medium	68.82	75.83	72.13	2.83	3.9
Low	12.78	20.25	16.44	2.27	13.8

[a] High, medium, and low concentrations were chosen to cover the standard calibration curve.
[b] C3 and C4 concentrations were measured 12-fold in the same assay.
[c] C3 and C4 concentrations were measured in 10 successive assays.

ranging from 20.9 to 335 mg/L. Similarly, linearity (n = 16; r = 0.993, $P < 0.001$) was observed in C4 analytical recovery over a range of 19.1 to 114.5 mg/L. The total recovery curves of C3 and C4 resulted in slopes of 1.016 and 1.021, respectively, and intercepts (−1.39 mg/L and −3.911 mg/L) not significantly different from 1 and 0 ($P > 0.05$), respectively. The mean percentages of recovery calculated reached 99.1% (SD 4.3%) for C3 and 92.1% (SD 9.3%) for C4.

Changes in C3 (663 samples) and C4 (657 samples) concentrations during lactation are shown in Fig. 3. Significantly different concentrations (mean ± SE mg/L) ($P < 0.001$) were observed for C3 among colostrum (n = 155; 199.32 ± 16.35 mg/L) transitional milk (n = 198; 57.71 ± 5.18 mg/L), and mature milk (n = 310; 30.36 ± 1.57 mg/L). A similar significant decrease ($P <$ 0.001) was observed for C4 concentration among colostrum (n = 159; mean = 113.42 ± 11.16 mg/L), transitional milk (n = 196; mean = 72.39 ± 4.98 mg/L), and mature milk (n = 302; 53.38 ± 3.61 mg/L) ($P <$ 0.05).

The decrease in C3 concentration however appeared to be sharper than for C4. This was confirmed by comparing the C3/C4 ratio, which revealed significant differences ($P < 0.001$) among colostrum (2.22; SD = 1.45; n = 152), transitional milk (0.94; SD = 0.50; n = 196), and mature milk ($P < 0.05$) (0.82; SD = 0.43, n = 302).

DISCUSSION

This study reports on the sequential assay of complement fractions C3 and C4 in human milk over the first 12 weeks of lactation. These assays were performed using an adapted immunonephelometry method. Wide ranges of calibration (from 11.56 to 370 mg/L for C3 and from 9.10 to 292 mg/L for C4) were obtained with a good reproducibility (CV <9% in the case of C3 and CV <14% for C4 in within- and between-run precision) and accuracy (linear recovery in dilution-overloading assays).

Although immunonephelemetry now stands as the reference routine technique for serum proteins quantification, RID was used in the few studies in the literature reporting on C3 and C4 levels in human milk (8–13). This choice had likely been prompted by milk's turbidity. However, after taking the precaution of preclearing the samples, we were able to develop efficient immunonephelemetric assays for these proteins in human milk. Nephelometry and RID have been reported to correlate well (21), yet the levels we report are slightly lower than those published previously. This could be due to the fact that we used a large population, comprising between 155 to 310 samples per stage of lactation, while previous reports rarely dealt with more than 30 samples. Another factor of variation could be the specificity of the antisera used, which differs between studies. The RID technique itself might yield overestimated concentrations since the long incubation time necessary (24–48 hr) may induce agarose/sample interactions leading to a cleavage of C3 and/or C4 (22). Apart from these technical details, the lower levels we obtained could be of ethnical origin since previously published studies examined the lactation of African (12) or Asian (13) women. Little infor-

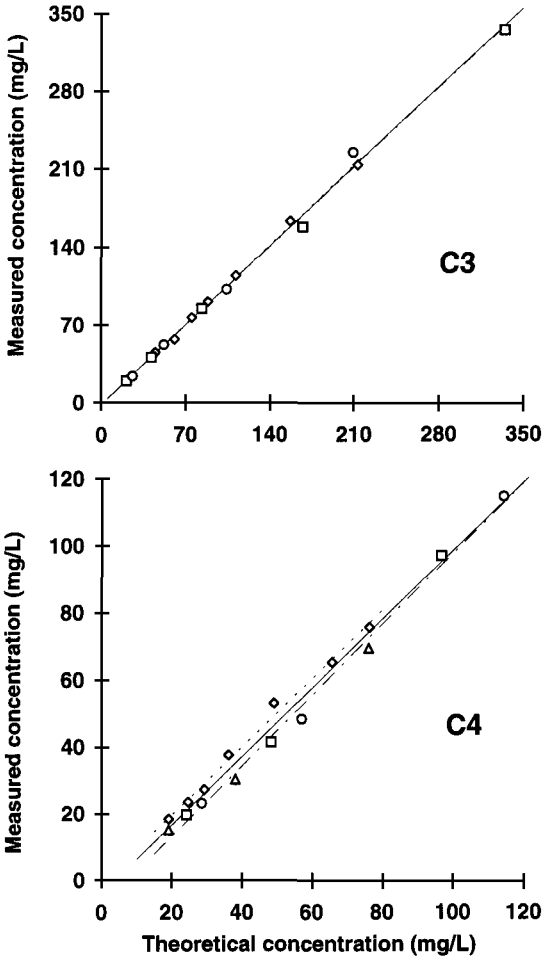

Fig. 2. Analytical recovery of milk C3 and C4 dilution assays (□, ○, △, ——); overloading assays (◇, . . .); linear regressions for total recovery (——).

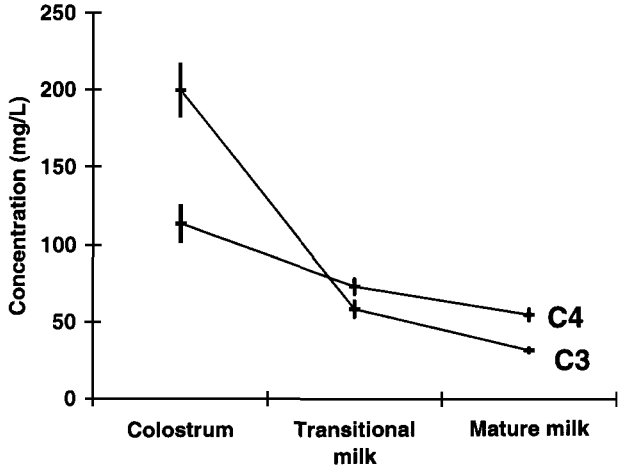

Fig. 3. Changes in C3 and C4 concentrations in human milk during lactation. Results are plotted as mean ± SE (□).

mation (10, 11) is available regarding the evolution of milk complement fractions in Caucasian women, which we examined. C3 and C4 concentrations in milk (30.36 ± 1.57 and 53.38 ± 3.61 mg/L, respectively) are much lower than in human serum [1230 ± 52 and 436 ± 23.6 mg/L (3)], and it could be argued that they merely originate from plasma suffusion. This hypothesis is supported by the higher concentrations noted in colostrum, the first secretion of the mammary gland, produced at a time when tight junctions are not fully closed (23). However, two characteristics indicate that local production should be considered. First, the milk/serum ratios [0.82 vs 4.46 (24)] of C3 and C4 are different, implying that some active passage is involved at least for C4. Second, the C3/C4 ratio varies as complement concentrations decrease over lactation. A possible source of C3 and C4 production could be milk mononuclear cells, the numbers of which are indeed highest in colostrum (25). These cells have been shown to express CD89 and could therefore be activated in the early days of lactation by the high levels of milk IgA present during this period (26). Another hypothesis is secretion by epithelial cells. This has been established for rat and human epithelial intestinal cell lines and appears to be dependent on interleukin secretion (27,28). The faster decrease in C3 could be explained by different rates of transport or secretion of these two molecules (11) or by different regulation mechanisms. The faster decrease in C3 levels could also be explained by the combined use of this complement fraction by the classical, alternate, and lectin pathways, C4 being used only in the first one. This would suggest that increased concentrations of complement activating factors are produced over lactation or that repeated irritation caused by factors such as frequent suckling or breast swelling induces inflammatory reactions in the lactating mammary gland.

CONCLUSIONS

These data extend and strengthen earlier smaller studies, demonstrating, furthermore, that key complement fractions are provided to the newborn throughout lactation at concentrations likely to sustain bacterial neutralization at a time when specific immune defenses are only beginning to develop.

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