

Endogenous Digitalis-Like Factors in Human Milk

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We measured the concentration of endogenous digitalis-like factors (EDLFs) in milk or colostrum of women during nursing on different days after delivery. EDLF concentrations were assayed by a solid-phase RIA involving anti-digoxin antibodies and by a radioreceptor assay (RRA) involving human placenta Na^+/K^+ -ATPase. The mean (SD) EDLF concentrations as measured by RIA were 35.6 (19.4) ng of digoxin equivalents per liter in milk samples ($n = 37$) and 61.3 (12.5) ng/L in colostrum samples ($n = 5$); the mean EDLF concentration as measured by RRA in milk samples ($n = 11$) was 573 (717) ng/L (range 0-2098). EDLF concentration in milk is greater than circulating concentrations in healthy adults but is comparable with serum concentration in the third trimester of pregnancy. In milk and serum samples ($n = 8$) collected at the same time, heating and (or) extracting with Sep-Pak C_{18} cartridges before the RIA produced significantly different EDLF values from those in untreated serum ($P < 0.001$) and milk ($P = 0.035$). EDLF in milk appeared to be not bound or weakly bound to milk protein, as indicated by the fact that boiling did not increase the digoxin-like immunoreactivity.

Additional Keyphrases: radioimmunoassay · radioreceptor assay · digoxin · colostrum

Endogenous factors with immunological properties and biological activity similar to digitalis drugs (endogenous digitalis-like factors, EDLFs) have been found in tissues and body fluids of animals and humans (1-5). EDLF concentrations are increased in amniotic fluid, cord blood, serum and urine of pregnant women, and neonates (2, 5). Recently, digoxin-like materials have been detected in saliva of pregnant women (6) and in human breast cyst fluids (7). The biochemical characteristics and physiological functions of EDLF are not well defined, but EDLF may play a role in the regulation of fluid volume and electrolytes in newborns and in the pathogenesis of arterial hypertension and eclampsia in pregnancy (2, 5). EDLF-like material may also regulate electrolyte composition of breast cyst fluids and indicate risk of developing breast cancer (7).

To establish whether EDLFs are normal constituents of human milk, we measured EDLF in milk or colostrum collected from women during nursing at different days after delivery by an RIA and by a radioreceptor assay (RRA).

Materials and Methods

RIA method. We assayed digoxin-like immunoreactivity by a previously reported solid-phase RIA (8, 9). Digoxin dissolved in a buffer containing 40 g of human serum albumin per liter is used as standard, ^{125}I -labeled digoxin is used as tracer, and a solid phase of antibody-coated test tubes is used for separating bound from free digoxin. The antiserum we used cross-reacts with digoxin by 35%, but negligibly (<0.1%) with testosterone, progesterone, cortisone, aldosterone, estradiol, estriol, cholesterol, dehydroepiandrosterone, cortisol, prednisone, prednisolone, and spironolactone (8, 9). Clinical results are reported as digoxin equivalents.

We used 0.2- or 0.4-mL sample volumes. Mean sensitivity (detection limit) for 10 separate experiments was 7.99 (SD 1.42) pg per tube, which corresponds to ~20 ng/L for 0.4 mL of sample. Mean between-assay CV for three different urine or plasma pools assayed over 10 months was 12.9%, 19.5%, and 16.4% (281.9 ± 36.4 , 120.0 ± 23.4 , and 57.4 ± 9.43 ng/L, respectively). The mean EDLF value for 38 healthy adult subjects was 16.1 ng/L (SD 7.8, range 0-40).

RRA method. We used a method (10-12) with ^{125}I -labeled digoxin as tracer and particulate membrane fractions of human placenta as the source of Na^+/K^+ -ATPase (Na^+/K^+ -transporting ATPase; EC 3.6.1.37). Placental membranes (0.6 g/L) were incubated with digoxin or milk samples [extracted on Sep-Pak C_{18} cartridges (Millipore-Waters, Milford, MA) and redissolved in assay buffer] for 5 h at 37 °C. After adding the tracer (^{125}I -labeled digoxin, 34 ng/L) and incubating the mixture for 2 h, we separated bound and free ligand by suction filtration through glass-fiber filters. The mean sensitivity was 49.2 (SD 20.2) pg/tube and the reproducibility, tested with one urine pool over four months, showed a CV of 29% (1500 ± 440 ng/L), with a within-run imprecision of 13.2%. The mean EDLF concentration measured in seven normal adult subjects was 344 (SD 432) ng/L.

Subjects and samples. Informed consent was obtained from all subjects.

By RIA we processed 59 specimens of human milk, 5 of colostrum, and 8 of serum collected from 24 women during nursing at different days after delivery (from 2 to 30 days); 11 samples of milk were also assayed by RRA. All milk and colostrum specimens were centrifuged ($2000 \times g$ for 10 min), and the lipidic phases on top were discarded.

By the RIA, we assayed the colostrum specimens and 37 milk specimens directly, without preliminary extraction or purification. We assayed the serum and milk specimens from eight of the nursing women after the samples were diluted threefold with distilled water,

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heated at 100 °C for 5 min, and (or) chromatographed with Sep-Pak C₁₈ cartridges, as previously described (13). We assayed 22 milk specimens after lyophilization-induced concentration: 2-mL milk samples were lyophilized and then reconstituted in 1 mL of water. For the dilution (parallelism) test, 2, 3, 4, 5, 6, and 7 mL from one pool of human milk were lyophilized and reconstituted with 1 mL of water; then 0.4 mL of each reconstituted solution was assayed.

For RRA, 2 mL of human milk was extracted on Sep-Pak C₁₈ cartridges before the assay, to remove interferences by proteins and ions (13) and to concentrate the sample. We evaporated the extracts under reduced pressure, dissolved the dry residues in 250 µL of incubation buffer, and then used 100 µL in the RRA.

Statistical analysis. Statistical analysis was carried out with a Macintosh SE personal computer and the 512+ Stat-View™ program (Brain Power, Inc., Calabasas, CA). Results are expressed as mean ± SD. Previously described computer programs were used to derive sample values and the index of sensitivity and to perform quality control for RIA and RRA (14, 15). The interpolation of the dose-response curves was calculated with a four-parameter logistic function (15).

Results

The mean (SD) EDLF concentrations measured directly (without purification or boiling) by RIA in human milk (n = 37) and colostrum (n = 5) were 35.6 (19.4) and 61.3 (12.5) ng/L, respectively. No correlation trend was found between EDLF values and time of milk collection after delivery. The mean EDLF concentration measured by RRA in 11 milk samples was 573 ng/L (SD 717, range 0–2098).

EDLF values for the eight samples of milk and serum collected simultaneously are given in Table 1. Analysis of variance (ANOVA) showed that the sample treatment before the assay did produce significantly different EDLF values both for serum ($P < 0.001$) and milk ($P = 0.035$). Purification produced a significant ($P < 0.05$) decrease of EDLFs in both serum and milk, perhaps because of partial recovery of EDLFs or removal of substances interfering with the RIA determination (15, 16). Boiling produced a significant increase of EDLF values for serum ($P < 0.001$) but not for milk, indicating that EDLFs are not bound or are weakly bound to milk proteins.

To improve the precision of the RIA, we assayed 22 milk samples that had been concentrated twofold by lyophilization; the mean EDLF concentration was 44.5 (SD 8.0) ng/L.

Results of the dilution test performed on a lyophilized and concentrated milk pool are reported in Figure 1. A linear relationship was found between the volume assayed and the immunoreactivity measured by RIA for as much as a fourfold concentration induced by lyophilization; the measurement was affected by higher concentrations.

Discussion

Our data indicate that endogenous factors with immunological and biological activity similar to digitalis drugs are normal constituents of human milk. We assessed the presence of substances with immunological properties similar to glycoside cardioactive drugs with a solid-phase RIA method (as cross-reactivity with anti-digoxin antibodies) and assayed digitalis-like biological activity by using the inhibition effect on ¹²⁵I-labeled digoxin binding to human placental Na⁺/K⁺-ATPase. As previously shown (12, 17, 18), EDLF values obtained by RIA and RRA are greatly different because of the different specificity and sensitivity of these two methods. However, both methods measured similarly greater EDLF concentrations (about twofold) in milk samples than in serum samples from healthy adult subjects [RIA: 35.6 (19.4) vs 16.1 (7.8) ng/L; RRA: 573 (717) vs 344 (432) ng/L].

EDLF concentrations in milk showed no trend for change during the first month of nursing, but more extended studies are needed.

The concentration of EDLF in milk and colostrum as measured by RIA without boiling or extraction was higher than that measured in serum of healthy adults but comparable with the serum concentration in the third trimester of pregnancy [55.1 (26.0) ng/L, n = 135 (5, 9, 19)]. Boiling serum samples before RIA significantly increased the measured EDLF concentration (Table 1) by disrupting the binding of these factors to plasma proteins (13, 16). In contrast, boiling did not affect EDLF values in human milk, suggesting that EDLFs are not bound or are weakly bound to milk proteins. Our data indicate that EDLFs are present in human milk not only in large amounts but also in free (unbound) form and therefore are bioavailable.

EDLFs may regulate electrolyte and fluid content of glandular cells and human milk by inhibiting the membrane Na⁺/K⁺-ATPase of breast cells, as suggested by Chasalow and Bradlow (7) for breast cyst fluids. Moreover, according to the hypothesis that EDLFs may actively regulate electrolyte-fluid balance in newborns and infants (5), EDLFs could be secreted (or concentrated) in human milk to provide infants with an exog-

Table 1. Effect of Sample Treatment on RIA Values for EDLF in Simultaneous Serum and Milk Samples*

Sample	Sample treatment			
	Direct assay	Boiling	Sep-Pak purif.	Boiling and purif.
Serum	47.5 (13.0)	141.4 (24.3)	19.6 (7.0)	34.3 (13.0)
Milk	60.6 (4.9)	65.3 (22.2)	35.0 (29.7)	40.1 (20.9)

* Mean (SD); n = 8; in nanograms of digoxin equivalents per liter. See text for statistical analysis.

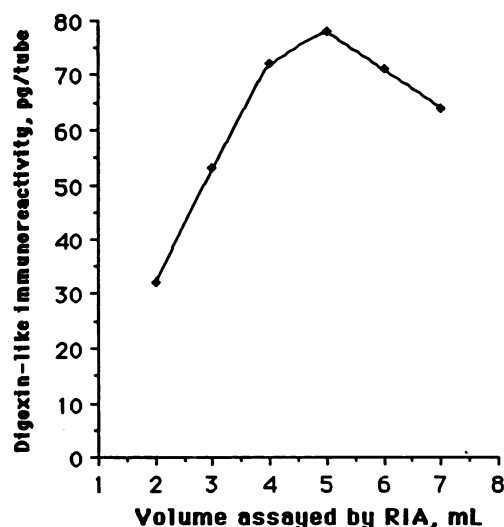


Fig. 1. Parallelism test

Different volumes (2, 3, 4, 5, 6, and 7 mL) of the same milk pool were lyophilized and reconstituted with 1 mL of water

enous contribution of digitalis-like factors to replace endogenous production, which tends to decrease after the first weeks of extra-uterine life (20-23). Further studies are necessary before physiological action in infants can be attributed to EDLFs in human milk. For example, it must be shown that milk EDLFs are not degraded and actually are absorbed by gastrointestinal tract in infants.

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