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Origin of Intact Lactoferrin and Its DNA-Binding Fragments Found in the Urine of Human Milk-Fed Preterm Infants. Evaluation by Stable Isotopic Enrichment[1]

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ABSTRACT. The origin of intact (78-kD) lactoferrin found in the urine of human milk-fed preterm infants was investigated using human milk containing proteins enriched with [^{13}C]leucine and [$^{15}N_2$]lysine or [$^2H_4$]lysine. Mothers of infants selected for the study were infused i.v. with [^{13}C] leucine and [$^{15}N_2$]lysine or [$^2H_4$]lysine to label milk proteins. The labeled milk was collected from each mother, pooled, fortified with a lyophilized human milk fraction, and fed to her preterm infant by continuous orogastric infusion for a period of 48 h. Urine was collected from each infant for 96 h. Intact lactoferrin (78 kD) and DNA-binding lactoferrin fragments (51 and 39 kD) were purified from the urine by affinity chromatography on columns of immobilized single-stranded DNA-agarose. The concentration and isotopic enrichment of the intact lactoferrin and DNA-binding fragments were determined separately after their isolation by high-performance reverse-phase (phenyl) chromatography. Mass spectral analyses indicated that the isotopic enrichment of the purified urinary lactoferrin was 87 to 100% of that in the labeled human milk lactoferrin. Similar results were obtained for the isolated DNA-binding lactoferrin fragments. The ratios of isotopically labeled leucine to lysine in the purified milk lactoferrins and urinary lactoferrins were similar for each mother/infant pair. Isotopically labeled lysine, added to the milk as free amino acid, was not incorporated into the purified urinary lactoferrin. These results demonstrate that undegraded (78-kD) lactoferrin of maternal origin is absorbed by the gut and excreted intact in the urine of preterm infants; nearly all of the urinary lactoferrin was of maternal origin. The possible immunoregulatory functions of the absorbed intact, maternal lactoferrin are discussed. (*Pediatr Res* 29: 243–250, 1991)

Abbreviations

APE, atom percent excess
GC-MS, gas chromatography-mass spectrometry
ssDNA, single-stranded DNA
HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
PAGGE, polyacrylamide gradient gel electrophoresis
HFBA, heptafluorobutyric acid

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Lactoferrin is a major constituent of human colostral milk and whey (1). This 78-kD secretory glycoprotein binds several different transition metal ions (2, 3), but is best characterized for its iron-binding properties and its association with host defense at mucosal surfaces (4). Perhaps more interesting are its postulated growth-promoting (5, 6) and immune-modulating properties (7–11). The structures of human hololactoferrin and apolactoferrin are known (12, 13), but their full biologic significance and mechanisms of action remain the subject of continued investigations.

Utilization of lactoferrin by the immature gastrointestinal system of preterm and term infants is poorly characterized. Infants fed human milk excrete higher levels of fecal lactoferrin than do infants fed bovine milk-based formulas (14–18). Fecal excretion, however, accounts for only a few percent (1–6%) of the lactoferrin fed (16, 17), and most fecal lactoferrin is degraded to lower molecular mass forms (19–21). Investigators have also reported higher levels of lactoferrin and lactoferrin fragments, free secretory component, and secretory IgA in the urine of infants fed human milk than in the urine of infants fed a bovine milk-based formula (21, 22). The molecular sizes of these immune factors are larger than normally filtered by glomeruli (23). The serum levels of lactoferrin are similar in formula- and human milk-fed preterm infants (0.8 to 0.9 μg/mL) (22). More recently, Scott (24, 25) has reported that the serum levels of lactoferrin in preterm infants are substantially higher than in term infants, and no difference was observed between term infants fed exclusively human milk and those fed bovine milk-based formula.

To determine the origin and quantify the relative urinary concentrations of intact lactoferrin and its fragments, simple and very efficient purification procedures are required. We have used immobilized DNA as an affinity method to purify human milk lactoferrin (26) as well as intact lactoferrin from the urine of human milk-fed preterm infants (27). We report here the stable isotope incorporation into human milk lactoferrin and its use to demonstrate the origin of intact lactoferrin, and of two relatively large (51- and 39-kD) lactoferrin fragments, present in the urine of human milk-fed preterm infants.

MATERIALS AND METHODS

Materials. L-[1-^{13}C]leucine and L-[2,6-$^{15}N_2$]lysine-HCl or L-[4,4,5,5-$^2H_4$]lysine-HCl (Merck Sharp and Dohme, Montreal,

Canada) were assayed by GC-MS and were found to contain 99 APE ^{13}C, 96 APE $^{15}N_2$, and 98 APE deuterium, respectively. The isotopically labeled leucine and lysine were dissolved aseptically and individually in 0.9% sodium chloride and potassium phosphate buffer (pH 7.2) to concentrations of 15 and 25 mg/mL, respectively. The isotopic solutions were verified to be sterile and pyrogen free by standard culture plate techniques and Limulus amebocyte lysate assays (Pyrogent, Mallinckrodt, St. Louis, MO), respectively.

Selection of lactating women. Two healthy, lactating mothers of preterm infants (27–28 wk gestation) participated at 6 wk postpartum. Informed written consent was obtained from each mother. The study was approved by the Institutional Review Board for Human Research at Baylor College of Medicine and the Clinical Investigations and Publications Committee of Texas Children's Hospital. The mothers were admitted to the Texas Children's Hospital General Clinical Research Center for 3 d. Medical histories and physical examinations were conducted before participation. The values for time postpartum, age, height, and weight of the subjects are shown in Table 1.

Maternal dietary intakes. Before admission, both women were interviewed by a research nutritionist to determine dietary intakes by 24-h dietary recall. The mothers were then given a controlled diet of protein and energy for 3 d. This diet consisted of commercially prepared formula and pudding (Sustacal, Mead Johnson, Evansville, IN) designed to provide protein intakes of 1.0 g/kg/d and energy intakes that equaled each individual's usual dietary intake determined from the dietary recall (Table 1). To promote compliance, additional protein-free fruits and vegetables were added to the diet in an amount <10% of the total daily energy intake. The subjects consumed four isocaloric, isonitrogenous meals at 0800, 1200, 1700, and 2100 h. Daily protein and energy consumption were determined from the differences in the weights of pre- and postmeal formula and pudding containers.

Maternal milk production. Maternal milk production was determined throughout the controlled dietary period. Milk samples were collected from both breasts in preweighed containers at 3-h intervals by mechanical pumping (Egnell, Cary, IL). Total daily milk production was calculated from the differences in the pre- and postweights of the milk collection containers during the 3-day study interval (Table 1). A weighed aliquot of milk was obtained from each milk expression and stored at −20°C until fortification and further analysis for protein, lactoferrin, and energy concentrations.

Incorporation of isotopically labeled amino acids into milk proteins. A primed, constant infusion of [^{13}C]leucine and [$^{15}N_2$]

Table 1. *Characteristics of mothers and infants evaluated in this study*

Characteristic	Subject 1	Subject 2
Mother		
Age (y)	36.1	30.4
Ht (cm)	161	165
Wt (kg)	68.3	65.5
Protein intake (g/kg/d)	1.0	1.0
Energy intake (kcal/d)	2085	2210
Milk production (g/d)	665	1467
Milk (fortified) lactoferrin concentration (mg/mL)*	1.89 ± 0.23	2.95 ± 0.20
Infant		
Gestational age (wk)	28	27
Birth wt (kg)	1.20	0.86
Age at study (d)	40	47
Body wt at study (kg)	1.47	1.55
Milk (fortified) intake during study (g/kg/d)	161	153

* Mean lactoferrin concentration (± SD) of fortified human milk (3 pools of milk/subject).

lysine (subject 2) or [$^{2}H_4$]lysine (subject 1) was administered to both volunteer mothers for 48 h. The isotopic solutions were administered i.v. via a peripheral hand vein as follows: [^{13}C]leucine and [$^{15}N_2$]lysine or [$^{2}H_4$]lysine, 5.5 and 3.2 μmol/kg, respectively, as priming doses; and [^{13}C]leucine and [$^{15}N_2$]lysine or [$^{2}H_4$]lysine, 4.6 and 3.2 μmol/kg/h, respectively, as the infusion dose. The latter solution was infused by means of a calibrated infusion pump (model 960, IMED Instruments, San Diego, CA) at a rate of 19 mL/h. Milk samples were collected before, during, and after the period of stable isotope infusion. Aliquots of the milk samples collected at 3-h intervals over a 48-h period were used to evaluate the isotopic enrichment of leucine and lysine in lactoferrin. Aliquots of each of the pooled, fortified, and labeled milk samples were removed for purification of lactoferrin and the determination of its isotopic enrichment by GC-MS analyses.

Preterm infant feeding of labeled human milk. The labeled milk samples, collected from each mother during the 48-h infusion, were pooled and fortified (30% of total nitrogen) with unlabeled lyophilized human milk powder prepared from our Human Milk Bank (28). Fortified human milk (29) was prepared daily by adding skim fractions (derived from pasteurized, lyophilized, mature, donor human milk) to fresh milk from each infant's mother. Free [$^{15}N_2$]lysine (200 μg/mL) or [$^{2}H_4$]lysine (100 μg/mL) was added to the fortified, labeled milks of subjects 1 and 2, respectively. The two preterm infants evaluated in this study were 28 wk gestational age at birth and showed appropriate growth for gestational age. They were free of congenital abnormalities, and cardiopulmonary, infectious, and gastrointestinal disorders. Each preterm infant was tolerant to complete enteral feedings by 15 d of postnatal life and was fed (at 6 wk of age) unlabeled fortified human milk for a minimum of 1 wk before the introduction of isotopically labeled human milk by continuous orogastric infusion (150 mL/kg/d) for 48 h. This particular feeding protocol has been discussed fully (29). During the remaining 48 h of the study period, the infants continued to receive fortified human milk.

Collection of preterm infant urine and quantitation of lactoferrin and protein. The preterm infant urine was collected in 8-h pools for a 96-h period, which commenced with the onset of feeding labeled, fortified human milk (20, 21). Urinary lactoferrin levels were quantified by routine ELISA methods (30). Polyclonal antibodies directed against human milk lactoferrin (Dako Corporation, Santa Barbara, CA) were immobilized on a plastic 96-well microtiter plate. The urine samples with (positive controls) and without added lactoferrin were first incubated with the primary, immobilized antibody. A serial dilution of purified human lactoferrin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was also included on each microtiter plate. The plates were then washed to remove unbound reagents before incubation with a second anti-human lactoferrin antibody conjugated to horseradish peroxidase. The color reaction was developed with hydrogen peroxide in citrate buffer containing O-phenylenediamine (Sigma Chemical Co., St. Louis, MO). Absorbance values were measured at 490 nm. Urinary protein levels were assayed by the method of Smith *et al.* (31) using the bicinchoninic acid protein reagent obtained from Pierce (Rockford, IL).

Purification of the urinary lactoferrin by single-stranded DNA-agarose affinity chromatography. Calf thymus ssDNA-agarose containing 0.61 mg DNA/mL gel (lot no. 71101) was obtained from Bethesda Research Laboratory, Gaithersburg, MD. It was packed into a 1.5-cm inner diameter column to a bed volume of 10 mL. The column was washed with water and then equilibrated with 20 mmol/L HEPES at pH 7.0 containing 3 mol/L urea. The flow rate was maintained at 30 mL/h. The separation procedure was carried out at room temperature (22 to 25°C). Trace amounts of [^{125}I]-labeled human lactoferrin (prepared by the lactoperoxidase method of Marchalonis (32) using a kit from Bio-Rad, Richmond, CA) were added to a 20-mL portion of

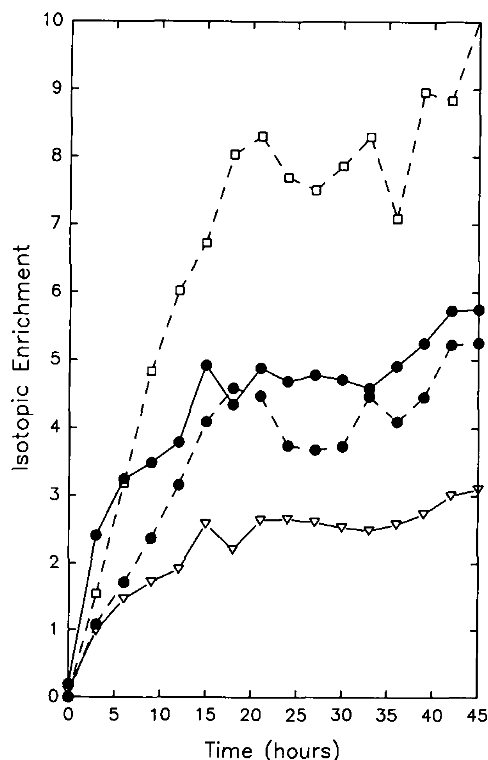

Fig. 1. Uptake of the stable isotopes [^{13}C]leucine (*filled circles*) and [$^{15}N_2$]lysine (*open triangles*) or [$^{2}H_4$]lysine (*open squares*) into purified human milk lactoferrin by subject 1 (*dashed lines*) and subject 2 (*closed lines*).

pooled preterm infant urine. After addition of urea to 3 mol/L and pH adjustment to 7.0, the urine sample was centrifuged (1000 × *g* for 10 min) and the supernatant was loaded onto the ssDNA-agarose column. The column was then washed with 30 mL of 20 mmol/L HEPES buffer (pH 8.0) containing 6 mol/L urea. Urea was subsequently eliminated by washing with three bed volumes of 20 mmol/L HEPES (pH 8.0) buffer. Elution with three bed volumes each of 0.2 mol/L and 0.5 mol/L NaCl resulted in the elution of nonlactoferrin proteins and lactoferrin, respectively. Fractions of 2 mL were collected for determination of UV absorbance at 280 nm (Beckman model DU-70, Beckman Instruments, Fullerton, CA) and radioactivity (Packard Autogamma 5500, Packard Instrument Co., Inc., Downers Grove, IL). To regenerate the ssDNA-agarose column, it was washed with 8 mol/L guanidine hydrochloride in 20 mmol/L HEPES (pH 8.0), followed by water. The purity of the isolated lactoferrin was evaluated by SDS-PAGGE and silver-staining, high-performance reverse-phase (phenyl) chromatography, high-performance ion-exchange (Mono-S) chromatography, and when possible, N-terminal amino acid sequence analyses (27, 33).

SDS-PAGGE of purified lactoferrin. The samples from the ssDNA-agarose column were concentrated (100- to 300-fold) using a Centricon-10 (Amicon, Danvers, MA) centrifugal concentrator (10 000 molecular wt cutoff). An aliquot of the concentrated sample (50 to 100 μL) was mixed with solubilizing buffer (50 μL 2% SDS with 3% mercaptoethanol) and heated in a boiling water bath for 90 s. Electrophoresis on a 10 to 20% polyacrylamide separating gel with 4.5% stacking gel was performed essentially according to the method of Laemmli (34). After electrophoresis, the gel was fixed with 10% acetic acid in 50% methanol, then silver stained, with minor modifications, according to the method described by Morrissey (35). Low molecular weight markers (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), including phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), α-lactalbumin (14.4 kD), and human milk lactoferrin (78 kD) standard (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), were used to determine the molecular mass of the purified urinary lactoferrin.

High-performance reverse-phase chromatography. An aliquot (50 μL) of the concentrated, purified lactoferrin sample obtained from ssDNA-agarose was applied to a Vydac TP219 (diphenyl) reverse phase column (4.6 mm × 25 cm, 5-μm particle size, 300-Å pore diameter; The Separations Group, Hisperia, CA) and eluted with a linear gradient of 44.5 to 52.2% solvent B in solvent A over 30 min (solvent A: 0.5% HFBA in Milli-Q (Millipore Corp., Bedford, MA) water; solvent B; 0.5% HFBA in 100% acetonitrile). The flow rate was 1 mL/min and protein elution was determined by UV absorbance at 280 nm (Beckman System Gold HPLC with a model 166 or 167 UV detector, Beckman Instruments).

Electrophoretic (Western) transfer and immunoblotting. SDS-PAGGE was performed under denaturing conditions except that reducing agents were eliminated during sample preparation. Approximately 5 μg protein were loaded to each well of the gel. Western transfer (36) was performed on nitrocellulose membranes (Bio-Rad) at pH 8.3 in 0.025 M Tris, 0.19 M glycine, 20% methanol buffer at 30 V for 16 h at room temperature (22 to 25°C). After 16 h, the voltage was increased to 60 V for 1 h. The nitrocellulose membranes were blocked for 1.5 h at 37°C with 0.1% gelatin (Bio-Rad) in 5 mM Tris-HCl, 0.15 M sodium chloride, pH 8.0. After blocking, the membrane was incubated for 1.5 h at 37°C with rabbit anti-human lactoferrin primary antibodies (purified Ig fraction) diluted in 5 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 0.05% Tween-20. After incubation with the primary antibody, the membrane was washed three times, then incubated for 1.5 h at 37°C with diluted goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Bio-Rad). Color development was performed using 4-chloro-1-naphthol with hydrogen peroxide in methanol.

Mass spectrometry. The ^{13}C, ^{15}N, or deuterium enrichments of free leucine and lysine, respectively, were determined after ion-exchange chromatography by GC-MS (37). Samples of pure lactoferrin, isolated from mothers' milk and their infants' urine, were hydrolyzed in 500 μL of 12 N HCl for 14 h at 110°C. The hydrolysates were evaporated to dryness, and converted to the n-propyl ester, *N*-heptafluorobutyramide derivative (38). GC-MS analyses of these derivatives were performed in triplicate on a Hewlett Packard HP-5988A quadrupole mass spectrometer (Palo Alto, CA) using the [M-HF]$^-$ ions produced by electron-capture negative ion GC-MS (39). Isotopic enrichment data are expressed as the molar ratio of isotopically labeled amino acid to unlabeled amino acid (×100) after correction for natural abundance as described previously (39).

RESULTS

Incorporation of [^{13}C]leucine and [$^{15}N_2$]lysine or [$^{2}H_4$]lysine into milk lactoferrin. Figure 1 shows the rate and extent of stable isotopic enrichment of maternal lactoferrin purified from aliquots of the milk collected at 3-h intervals during the 48-h period of isotope infusion. All of the labeled milk collected from each subject during the 48-h period of isotope infusion was pooled and fortified with human milk powder. Enzyme-linked immunoassays indicated that these fortified pools contained 1.89 and 2.95 mg/mL lactoferrin (Table 1). The [^{13}C]leucine and [$^{2}H_4$] lysine enrichments of lactoferrin in the pooled, fortified human milk fed to infant 1 were 3.52 ± 0.01 and 4.65 ± 0.04, respectively. The [^{13}C]leucine and [$^{15}N_2$]lysine enrichments of lactoferrin in the pooled, fortified human milk fed to infant 2 were 3.23 ± 0.008 and 1.65 ± 0.057, respectively.

Lactoferrin in the urine of human milk-fed preterm infants. Urine samples (8-h pools) collected from the two infants contained from 0 to 243 μg of lactoferrin per mL (Table 2). Because the urines were diluted to varying degrees during the collection process, these values do not reflect actual urine concentrations.

Table 2. *Characteristics of human milk-fed preterm infant urines*

Urine pool no.	Protein concentration (mg/mL)	Volume (mL)	Total protein (mg)	Lactoferrin (μg/mL)	Total lactoferrin (mg)
Infant 1					
1	3.14	50	157	10.38	0.519
2	1.67	9	15	0.00	0.000
3	4.58	70	320	1.45	0.101
4	4.69	50	235	15.45	0.773
5	1.98	105	207	3.73	0.392
6	3.56	40	142	2.55	0.102
7	2.80	70	196	21.39	1.497
8	3.13	60	188	14.55	0.873
9	3.02	70	211	30.31	2.123
10	1.71	120	205	24.42	2.930
11	3.19	80	255	10.18	0.814
12	3.21	45	145	8.79	0.396
Total		769	2.28 g		10.5
Infant 2					
1	4.35	48	209	1.94	0.093
2	4.00	60	240	16.13	0.968
3	4.13	55	227	36.03	1.982
4	4.59	58	266	74.50	4.321
5	1.78	49	87	15.27	0.748
6	3.56	47	167	44.31	2.083
7	4.26	28	119	242.95	6.803
8	2.26	129	292	97.26	12.547
9	3.15	18	57	31.76	0.572
10	3.22	28	90	1.30	0.036
11	3.04	53	161	6.89	0.365
12	2.75	81	223	18.34	1.486
Total		654	2.14 g		32.0

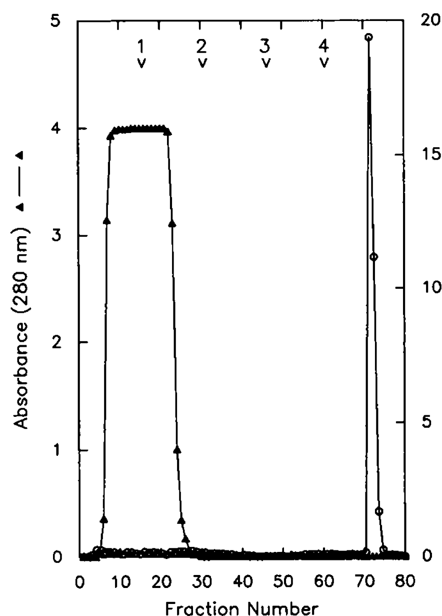

Fig. 2. Isolation of intact lactoferrin and DNA-binding lactoferrin fragments found in the urine of preterm infants fed human milk by affinity chromatography on ssDNA-agarose as described in Materials and Methods.

The total urinary outputs of lactoferrin, however, are provided in Table 2.

Purification and characterization of the urinary lactoferrin. The profile shown in Figure 2 illustrates the efficiency of ssDNA affinity columns for the one-step purification of intact lactoferrin and two major lactoferrin fragments from the urine of human milk-fed preterm infants. The majority of urinary protein detected by absorbance at 280 nm was eluted unretained in the

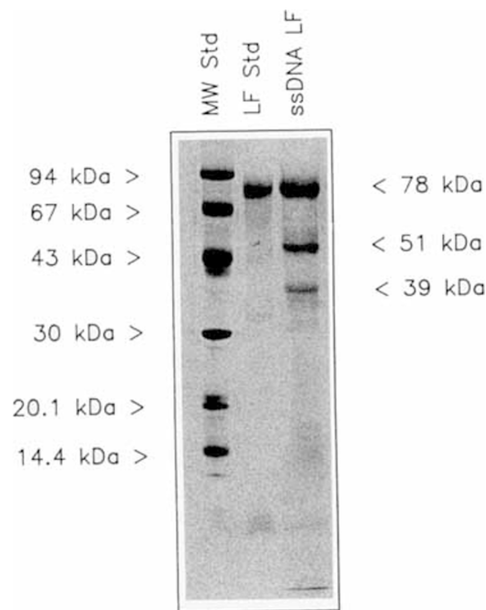

Fig. 3. SDS-PAGGE (10–20%) of affinity-purified urinary lactoferrin under denaturing conditions. The migration patterns of the purified proteins were visualized by silver-staining. *Lane 1*: calibrator proteins for the estimation of molecular mass (phosphorylase b, 94 kD; serum albumin, 67 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 20.1 kD; and α-lactalbumin, 14.4 kD). *Lane 2*: purified lactoferrin obtained commercially. *Lane 3*: urinary lactoferrin purified by affinity chromatography on ssDNA-agarose (intact 78-kD protein, 51-kD fragment, and 39-kD fragment).

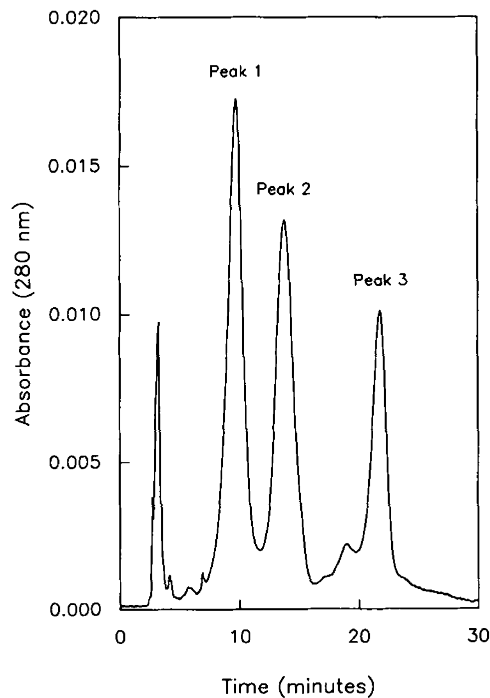

Fig. 4. Resolution of intact urinary lactoferrin and DNA-binding lactoferrin fragments (39 and 51 kD) by high-performance reverse-phase chromatography. Urinary lactoferrin and DNA-binding lactoferrin fragments were purified from urine by affinity chromatography on ssDNA-agarose before analysis on phenyl reverse-phase columns using HFBA as the ion-pairing reagent. *Peaks 1-3* represent the 39-kD fragment, 51-kD fragment, and intact 78-kD lactoferrin, respectively (see text). The profile shown is representative of 35 similar experiments. Details are provided in Materials and Methods.

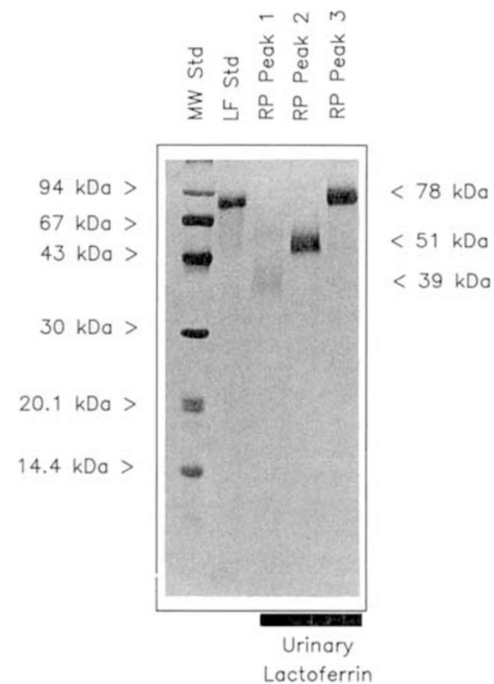

Fig. 5. Molecular mass evaluations and identification of intact urinary lactoferrin and the DNA-binding lactoferrin fragments isolated by affinity chromatography on ssDNA-agarose and resolved by high-performance reverse-phase chromatography. SDS-PAGGE of the affinity purified lactoferrin and lactoferrin fragments was performed after resolution of these lactoferrins by high-performance reverse-phase chromatography as described in Figure 4. *Lane 1* shows the migration pattern of the calibrator proteins used for estimation of molecular weight. *Lane 2* shows the electrophoretic migration properties of human milk lactoferrin obtained from Jackson Immunoresearch Laboratories. *Lane 3* illustrates the 39-kD lactoferrin fragment present in peak 1 of the high-performance reverse-phase chromatographic elution profile shown in Figure 4. *Lane 4* shows the electrophoretic migration profile of the 51-kD lactoferrin fragment present in peak 2 of the high-performance reverse-phase chromatogram (Fig. 4). *Lane 5* shows the electrophoretic migration profile of the intact lactoferrin present in peak 3 of the high-performance reverse-phase chromatogram (Fig. 4).

flow-through fractions. The elution position of the absorbed urinary lactoferrin was typically not detectable by its absorbance at 280 nm alone. Thus, in separate experiments, the elution position and recovery of urinary lactoferrin were verified by the addition of trace quantities (<5 pg) of [^{125}I]-labeled lactoferrin to test aliquots of preterm infant urines. Under the conditions specified, the recovery of lactoferrin was routinely (n = 50) greater than 90% and often near 100%.

SDS-PAGGE of the urinary lactoferrin purified by ssDNA-agarose affinity chromatography revealed the presence of proteins of 78, 51, and 39 kD when the gel was silver-stained (Fig. 3). The presence of intact lactoferrin (78 kD) was confirmed using polyclonal antisera to immunoblot the electrophoretic gels. Lower molecular weight lactoferrin fragments were also detectable. Using the more sensitive (detection limit 50 to 100 pg) lactoperoxidase and gold conjugate immunoblot detection systems as described previously (30, 40), these lower molecular mass (39- and 51-kD) proteins were also identified as immunoreactive lactoferrin. To determine their possible separate metabolic origins, the intact lactoferrin and its two DNA-binding fragments required separation before mass spectral analysis.

Separation of urinary lactoferrin fragments from intact lactoferrin. The efficient micropreparative separation of the intact urinary lactoferrin from the 51- and 39-kD urinary lactoferrin fragments was achieved only by means of high-performance reverse-phase chromatography on phenyl-derivatized silica columns employing HFBA as the ion-pairing reagent. The profile shown in Figure 4 illustrates the elution pattern of urinary lactoferrin and lactoferrin fragments from one of the two infants fed labeled milk (the other preterm infant evaluated in this study produced urines that contained insufficient quantities of lactoferrin to justify further separation of fragments by this method). SDS-PAGGE and immunoblot analyses with anti-lactoferrin antibodies were used to confirm the identity of the three reverse-phase peaks. The SDS-PAGGE pattern in Figure 5 shows the intact lactoferrin as well as the two major DNA-binding fragments after their collective isolation by affinity chromatography on ssDNA-agarose and separation by high-performance reverse-phase chromatography. Immunoblot analyses confirmed the immunoreactivity of these proteins and led to the identification of the three reverse-phase peaks as the 39-kD lactoferrin fragment, the 51-kD lactoferrin fragment, and intact lactoferrin, respectively. The isolated urinary lactoferrin (and fragments) recovered from each of the 12 8-h urine pools were evaluated by GC-MS for isotopic enrichment.

Isotopic enrichment of urinary lactoferrin and its fragments. The profiles shown in Figure 6 demonstrate the rate and extent to which isotopically labeled lactoferrin appeared in the urine collected from each of the two preterm infants fed the labeled, fortified human milk. In the case of subject 2 (Fig. 6*A* and *B*), separate data are shown for the intact lactoferrin and the 39- and 51-kD lactoferrin fragments isolated by reverse-phase chromatography. The *horizontal line* across the top of each profile indicates the isotopic enrichment of the lactoferrin present in the labeled, fortified milk that was fed to each of the two infants. The rate and extent of isotopic enrichment of the intact urinary lactoferrin (78 kD), the 39-kD urinary lactoferrin fragment, and the 51-kD urinary lactoferrin fragment were quite similar. In each case, the isotopic enrichment of [^{13}C]leucine (Fig. 6*A*) and [$^{15}N_2$]lysine (Fig. 6*B*) increased to a level equal to the isotopic

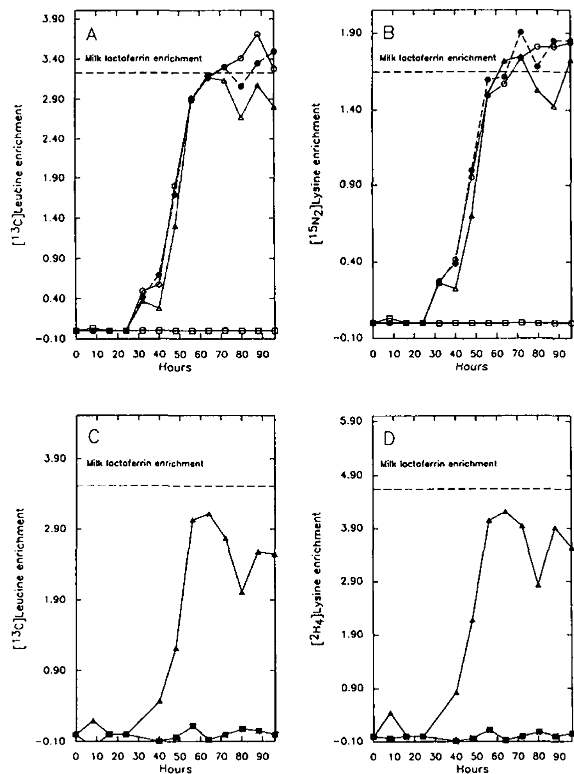

Fig. 6. Appearance of urinary lactoferrin enriched with the stable isotopes. Isotopic enrichments were determined as a function of time (h) after introduction of labeled milk as described in Materials and Methods. The rate of $[^{13}C]$leucine (*panel A*) and $[^{15}N]$lysine (*panel B*) accumulation in the preterm urinary lactoferrin (subject 2) is shown for the intact (78-kD) protein (*open triangles*), the 39-kD fragment (*open circles*), and the 51-kD fragment (*filled circles*). *Panels C* and *D* show the rate of $[^{13}C]$ leucine and $[^2H_4]$lysine accumulation in the preterm urinary lactoferrin (*closed triangles*; intact lactoferrin and fragments) isolated from subject 1. The level of specific isotopic enrichment for lactoferrin purified from the human milk actually fed is shown by the *dashed horizontal line* near the top of each profile. Data points shown along the bottom of each profile indicate that $[^{15}N_2]$lysine (*filled squares*) and $[^2H_4]$lysine (*open squares*), added as the free amino acid to the pooled, fortified milks from subjects 1 and 2, respectively, were not incorporated into the isolated urinary lactoferrin.

enrichment of the labeled lactoferrin in the fed milk; we observed comparable (87%) degrees of isotopic enrichment in the intact urinary lactoferrin isolated from the urine of infant 1 (Fig. 6*C* and *D*). The maternal origin of the urinary lactoferrins from each preterm infant was established by comparison of the ratios of isotopically labeled leucine to lysine in the isolated milk and urinary lactoferrins. The ratio of $[^{13}C]$leucine to $[^{15}N_2]$lysine in the purified milk lactoferrin isolated from subject 2 (1.957) was similar to that in the purified urinary lactoferrin from her preterm infant (1.866). The ratio of $[^{13}C]$leucine to $[^2H_4]$lysine in the purified milk lactoferrin isolated from subject 1 (0.756) was essentially the same as the ratio of these amino acids found in the purified urinary lactoferrin from her infant (0.743). The possible endogenous synthesis of lactoferrin by the infant and, in particular, its appearance in the urine, was addressed by the addition of $[^{15}N_2]$lysine (subject 1) or $[^2H_4]$lysine (subject 2) as the free amino acid to the pooled, fortified milks immediately before feeding. Neither $[^{15}N_2]$lysine (subject 1), nor $[^2H_4]$lysine (subject 2) was detected in the purified urinary lactoferrins from either preterm infant (Fig. 6).

Relationship of urinary lactoferrin to total urinary protein. The concentrations of total urinary protein collected over the 96-h period were similar for the two human milk-fed preterm infants studied during this investigation (Table 2). In contrast, however,

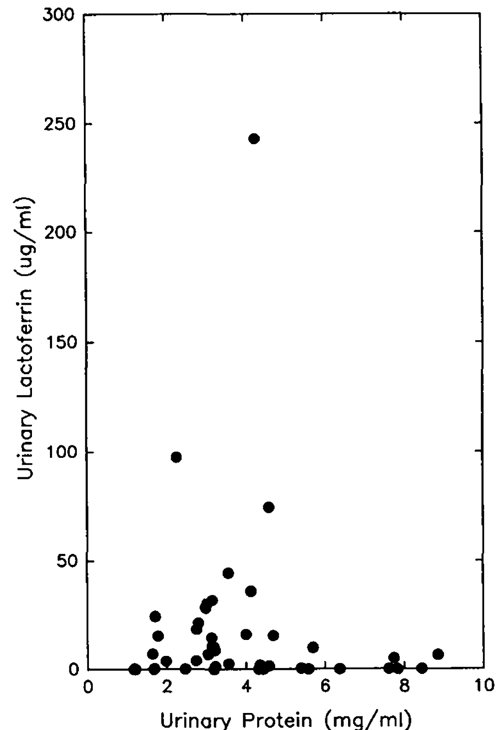

Fig. 7. Relationship between urinary lactoferrin concentrations and total urinary protein concentrations. Lactoferrin concentrations in the diluted urines were determined using enzyme-linked immunosorbent sandwich assays as described in Materials and Methods.

the quantities (total output) of urinary lactoferrin measured in these infants were quite different and varied significantly over the 96-h collection period (Table 2). The urinary lactoferrin concentrations were not related to total urinary protein concentrations (Fig. 7). Urinary lactoferrin and protein in the collected urine pools are given as concentrations in Figure 7; these concentrations reflect varying degrees of dilution associated with the routine urine collection procedures (29). The data presented in Table 2 for total urinary lactoferrin and protein outputs indicate that the lack of correlation between lactoferrin and protein was independent of the collection procedure (*i.e.* dilution).

DISCUSSION

Serum levels of lactoferrin in both preterm and term infants reportedly do not vary with diet (*i.e.* human milk *versus* bovine milk-based formula) (22, 24). As a result, the origin of biologically significant quantities of lactoferrin in the urine of preterm infants fed human milk has been the subject of much discussion (*e.g.* 21, 22). Because fecal excretion of lactoferrin accounts for only 1 to 6% (15–17) of the dietary lactoferrin in preterm infants, it has been suggested that much of the remaining lactoferrin is digested (14–18). This assumption remains untested. We have now demonstrated that nearly all of the intact lactoferrin in the urine of premature infants fed human milk is of maternal origin.

The similar ratios of labeled amino acids in the 78-kD lactoferrin purified from milk and preterm infant urine indicated that the labeled, intact lactoferrin excreted in urine by these infants was definitely of maternal origin; furthermore, the absolute levels of enrichment of $[^{13}C]$leucine and $[^{15}N_2]$lysine or $[^2H_4]$lysine in the urinary lactoferrin demonstrated that most (87 to 100%) of the recovered lactoferrin was of maternal origin. This conclusion is supported by our observation that the isotopically labeled free amino acid $[^2H_4]$lysine or $[^{15}N_2]$lysine (added directly to the expressed, pooled milk samples) was not incorporated into either the intact urinary lactoferrin or its isolated fragments. Therefore, if endogenous lactoferrin was produced, it did not appear in their urine.

The physiologic mechanisms that account for the uptake of intact lactoferrin by the gastrointestinal tract and its subsequent renal excretion were not addressed by these studies. The delay (>24 h) between the introduction of labeled lactoferrin and its appearance in urine was significant. The lack of a detectable association between urinary lactoferrin and total protein (either concentrations or total output) noted in the present study, and the significant correlations between the amounts of urinary immunologic factors excreted by preterm infants observed in previous studies (20) suggest that specific mechanisms are responsible for the presence of these proteins in urine. The question of selective protein excretion by infants who are fed human milk requires further analyses.

The urinary lactoferrin fragments are of considerable interest. These fragments, observed in at least eight different urine pools, were separated only under denaturing conditions *in vitro*. Under nondenaturing conditions, neither the 39- nor 51-kD fragments were detected. The origin, nature, and regulation of the lactoferrin cleavage event(s) are not known. The indicated fragments were not detected in the milk of these mothers, in the milk of 10 other mothers that we studied (unpublished observations), or of others studied previously (21). These lactoferrin fragments that bind to DNA also are not detected in the milk of other mammals (bovine and porcine) we have studied (unpublished observations). It is therefore likely that these fragments arise from a limited or specific proteolysis either in the gastrointestinal tract, in the systemic circulation, and/or during filtration and excretion in the urine. Preliminary N-terminal sequence analysis of the purified 39- and 51-kD fragments suggests that they represent portions of the N-lobe and the intact C-lobe of lactoferrin, respectively. The appearance of these two lactoferrin fragments only under denaturing conditions and always in a near 1:1 stoichiometry suggests that they are not totally separated *in vivo*, but complexed in a manner that sustains their association (41). The preserved DNA-binding properties of the intact lactoferrin and lactoferrin fragments imply that the DNA-binding domain of the lactoferrin molecule is relatively resistant to further proteolytic degradation.

The physiologic significance to preterm infants of absorbed, undegraded lactoferrin remains to be established. Its uptake may proceed by nonspecific macromolecular absorption (42) or by way of association with specific lactoferrin receptors (43–49). Bennett and coworkers (7–9) have shown that the DNA-binding functions of lactoferrin affect intercellular communications among circulating lymphocytes, neutrophils, and granulocytes. Although the tertiary structure and function of the urinary lactoferrin has not yet been documented, the ability of lactoferrin to be absorbed in its intact form suggests that it may act in a regulatory capacity to influence the infant's developing immune system. The abilities of two relatively large (39- and 51-kD) DNA-binding fragments to resist further degradation and to enter the circulatory system otherwise intact suggest that they also may act in a regulatory capacity. These findings suggest that at least in preterm infants who are fed human milk, lactoferrin may play a broader biologic role than has been previously appreciated. Indeed, human milk lactoferrin does not appear to be a transient immunoprotective protein localized to the gastrointestinal tract. The precise developmental period that may ultimately preclude the absorption and (or) renal excretion of intact lactoferrin (*e.g.* term infants) is currently under investigation.

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