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Isolation and Characterization of Rhesus Monkey Milk Lactoferrin

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ABSTRACT. Rhesus monkey milk lactoferrin was isolated and its characteristics compared with those of human milk lactoferrin in order to assess the feasibility of using the rhesus monkey as an animal model for the study of iron absorption from milk. Monkey lactoferrin was isolated from pooled monkey milk by two chromatographic steps. Concentration of lactoferrin in milk, determined by rocket immunoelectrophoresis, demonstrated similar concentrations in both human and monkey milk, 1–2 mg/ml. Immunodiffusion of lactoferrins from several species using an antibody raised to money lactoferrin resulted in a cross-reaction only with monkey and human lactoferrin. Lactoferrins from cow, sheep, goat, dog, and rat milk were not recognized by the antibody. Amino acid analysis of monkey lactoferrin showed a composition very similar to human lactoferrin, as well as a similarity in the unusual amino acid sequence at the N-terminal of the protein. The carbohydrate moiety of monkey lactoferrin was investigated and shown to contain monosaccharides in similar proportions to those reported for human lactoferrin. In our opinion, the rhesus monkey is a promising model for the study of the role of lactoferrin in iron absorption in the infant, as well as of the other proposed actions of lactoferrin. (*Pediatr Res* 20: 197–201, 1986)

It has been documented that the absorption of iron from human milk is more efficient than from infant formulas based on cow's milk or soy protein (1, 2). The high degree of iron absorption from breast milk is manifested in a low incidence of iron-deficiency anemia among breast-fed infants. In contrast, infants consuming cow's milk formula without supplemental iron display a higher incidence of iron deficiency during the early months of life (3).

This high iron absorption from human milk is thought to be due to a higher bioavailability of the trace element from human milk. Despite the fact that the iron content of human milk and cow's milk are similar (4, 5), iron absorption from human milk has been observed to be three to five times higher than from cow's milk in both infants and adults (1, 2). Lactoferrin, the major iron-binding protein in human milk, has been implicated as a possible factor contributing to the high bioavailability of iron. Lactoferrin is similar in several physical and chemical properties to transferrin and has been shown to be present in various exocrine secretions (nasal, lacrimal, vaginal, bronchial) (6), in pancreatic juice and in specific granules of neutrophils (7). Both lactoferrin and transferrin can bind two ferric ions with high affinity. The binding constant of lactoferrin for iron is $\sim 10^{30}$ with iron not being completely released until the pH is lowered to 2 (8). This high affinity for iron is thought to provide a mechanism for a bacteriostatic effect by lactoferrin since iron is a required nutrient for many microorganisms. It has been demonstrated in an *in vitro* system that lactoferrin can inhibit the growth of several strains of bacteria, including *Escherichia coli* and *Staphylococcus aureus* (9). This effect is abolished if the protein is saturated with iron, indicating it is the iron-binding ability of lactoferrin that gives it the bacteriostatic property.

In vitro data show lactoferrin to be relatively resistant to proteolysis and low pH (10, 11) and there is evidence for the survival of intact lactoferrin during passage through the gastrointestinal tract of the infant (12, 13). These unique characteristics provide additional support for a bacteriostatic role for lactoferrin in the infant.

Lactoferrin has also been suggested to play a role in the immune system. The protein has been shown to bind specifically to human monocytes (14), polymorphonuclear leukocytes (15), and macrophages (16) and may be involved in several aspects of the inflammatory response.

In the present study we have investigated the use of an animal model in studying the role of lactoferrin in iron absorption since ethical considerations prevent the use of human infants in studies involving radioisotopes or necessitating the use of tissue samples. It has been shown previously that lactoferrins from human and bovine milk have different abilities to donate iron to human mucosa in an *in vitro* system (17). Therefore, it is essential to study lactoferrins with similar characteristics to the human protein in order to draw parallels to the role lactoferrin may play in iron absorption in the human.

In contrast to most species, lactoferrin concentration in both human and monkey milk is quite high, in the range of 1–2 mg/ml (18, 19). Monkey lactoferrin was isolated and its chemical and physical characteristics investigated in order to assess the feasibility of using the rhesus monkey as a model in the study of iron absorption from milk.

MATERIALS AND METHODS

Isolation of monkey lactoferrin. Pooled monkey milk (75 ml) was obtained from the California Primate Research Center, University of California, Davis, and was centrifuged at 15,000 × *g* for 30 min. The fat layer and casein pellet were removed and the resulting whey was made 2 M with ammonium sulfate, stirred for 20 min, and centrifuged at 10,000 × *g* for 30 min. The lactoferrin containing supernatant was brought to 75% saturation (4.4 M) with ammonium sulfate and centrifuged for 30 min at 10,000 × *g*. The resulting pellet was dissolved in 0.05 M Tris-HCl, pH 9.2; iron was added to saturate the lactoferrin (2 mol ferrous ammonium sulfate per mol lactoferrin) and sodium bicarbonate added to promote iron-binding to the protein (1 mol bicarbonate per mol iron) (20). The resulting solution was dialyzed against the Tris buffer until its ionic strength was identical to that of the buffer. This crude lactoferrin solution was chromatographed on a DEAE-Sephadex A-25 ion exchanger (15 × 2

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cm) in 0.05 M Tris-HCl, pH 9.2, employing a linear gradient of 0–0.5 M NaCl (400 ml plus 400 ml). The lactoferrin peak, identifiable by its pink color, was pooled, dialyzed against the starting buffer, and applied to a Heparin-Sepharose affinity column (6 ml), purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The column was eluted with a stepwise gradient of the same buffer containing NaCl (0–1.0 M) (21).

Purity of the monkey lactoferrin. Purity of the isolated monkey lactoferrin was assessed by polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels in 0.9 M acetic acid containing 6 M urea. Electrophoresis was run toward the cathode at 2 mA per gel for 4 h. Gels were stained with Coomassie Blue.

Physical and chemical characterization. The concentration of lactoferrin in monkey milk was determined by rocket immunoelectrophoresis (22) in 1% sodium borohydride treated agarose gels in 0.025 M Tris/Tricine buffer, pH 8.6 (Bio Rad Laboratories, Richmond, CA) using an antibody raised to the purified monkey lactoferrin. The agarose was obtained from Bio Rad Laboratories and treated to eliminate charged groups by the method of Lönnerdal and Låås (23). Antibody to monkey lactoferrin was raised in rabbits by Antibodies, Inc. (Davis, CA) using six injections of lactoferrin in Freund's complete adjuvant. Electrophoresis was carried out for 3 h at 300 v and maximum current. The plate was washed and stained as described (24). Standards of known monkey lactoferrin concentrations were used to determine lactoferrin concentration in the milk samples. Concentrations in human milk were determined by the same method using antibody to human lactoferrin which was purchased from Dako Immunoglobulins (Copenhagen, Denmark). Human lactoferrin was prepared from pooled human milk obtained from healthy lactating donors.

Immunodiffusion was performed to investigate cross-reactivity of lactoferrin from several species. Monkey lactoferrin antibody was placed in the center well of a 1% agarose gel surrounded by wells containing milk samples from several species (human, monkey, cow, sheep, goat, dog, rat). Milk was obtained from healthy animals at the U.C. Davis animal facilities. The plate was left in a humid chamber for 3 days to allow diffusion. The plate was then processed as described above.

Amino acid analysis was performed following hydrolysis with hydrochloric acid in a sealed tube at 110° C for 24 and 48 h with performic acid oxidation for cysteine and methionine determination. Analysis was performed on a Durrum Model D-500 amino acid analyzer in 0.2 M sodium citrate with β-thienyl alanine as the internal standard (25, 26). The amino acid sequence of the N-terminus of monkey lactoferrin was determined on a Beckman 890M sequencer using 0.1 M quadrol containing polybrene. Identification was obtained using high-performance liquid chromatography by the method of Bhown *et al.* (27) with confirmation by thin-layer chromatography by the method of Kulbe (28).

The carbohydrate component of monkey lactoferrin was investigated using colorimetric assays for the monosaccharides reported to be present in human lactoferrin (29). Total hexose was determined with the anthrone method (30). Mannose, galactose, and fucose were quantified as reported by Finch *et al.* (31) after acid hydrolysis in 2 N HCl at 100° C for 1 h. N-acetyl glucosamine was determined after acid hydrolysis as described by Gatt and Berman (32), and N-acetyl neuraminic acid was quantified by the method of Schauer (33).

RESULTS

Physical and chemical characteristics of monkey lactoferrin were compared to those of human lactoferrin after purification of the protein from pooled monkey milk. By two chromatographic steps pure lactoferrin was obtained from monkey milk; DEAE-Sephadex chromatography gave a preliminary separation (Fig. 1), while final purification was achieved by Heparin-Sepharose affinity chromatography (Fig. 2). Lactoferrin eluted with

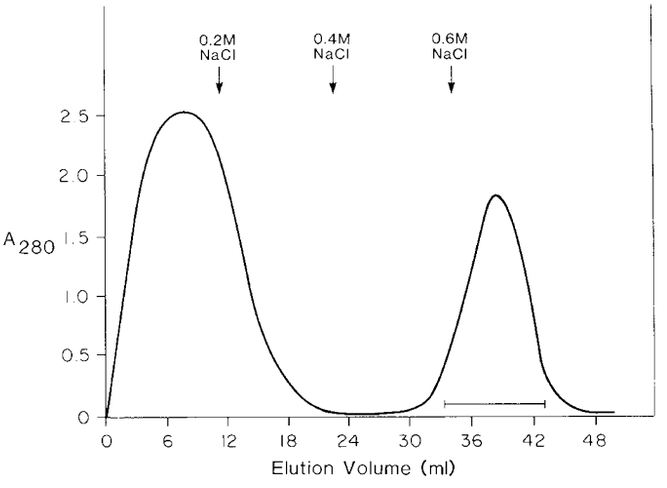

Fig. 2. Purification of lactoferrin by heparin-Sepharose affinity chromatography. The pure lactoferrin eluted with 0.6 M NaCl.

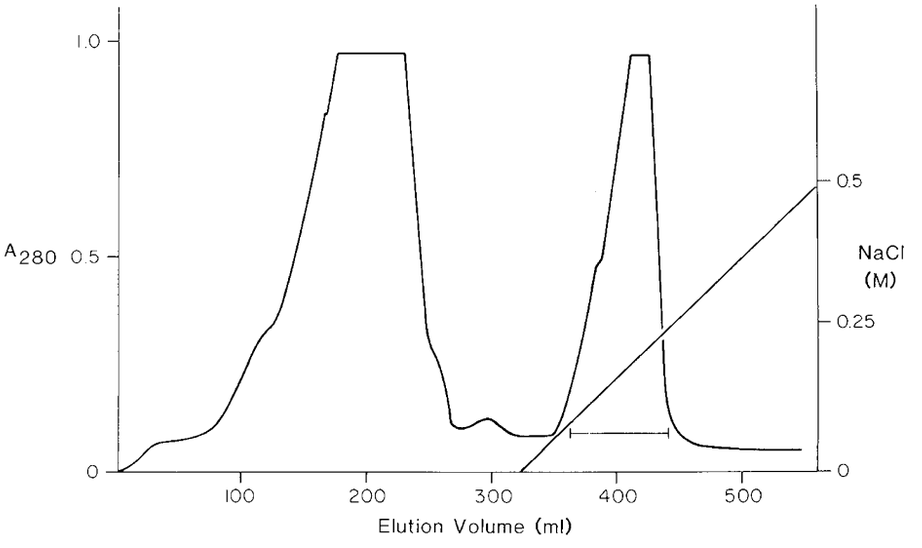

Fig. 1. Ion exchange chromatography on DEAE-Sephadex A-25. The lactoferrin peak is identified by the *bar*.

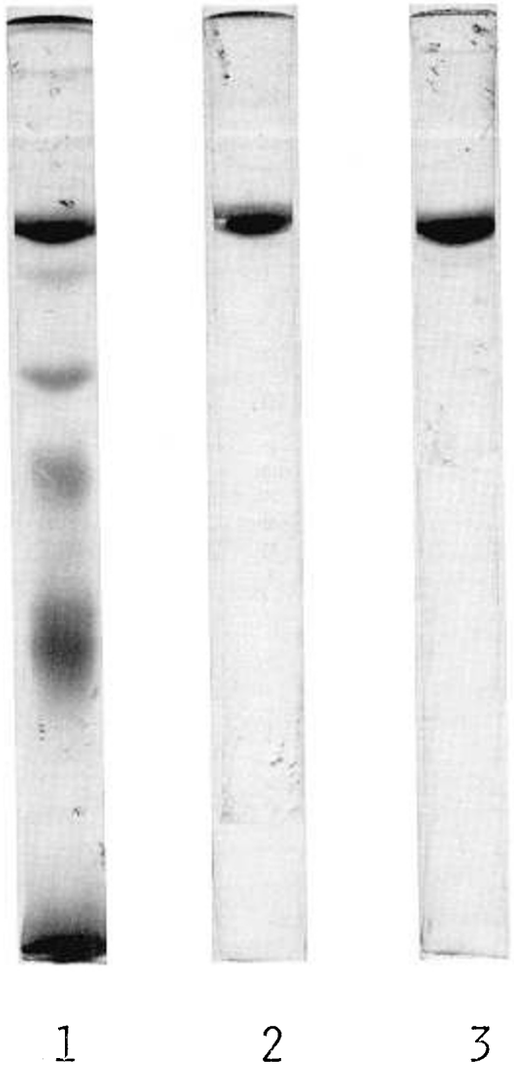

Fig. 3. Polyacrylamide gel electrophoresis of *1*, monkey whey, *2*, pure monkey lactoferrin, *3*, pure human lactoferrin.

Table 1. *Concentration of lactoferrin in human and monkey milk as determined by immunoelectrophoresis*

	Lactoferrin concentration (mg/ml)	
	Monkey milk	Human milk
	1.52	1.88
	2.00	1.88
	1.76	1.62
	1.88	1.91
	1.50	1.82
$\bar{x} \pm SD$	1.73 ± 0.22	1.82 ± 0.12

0.6 M NaCl. The resulting pale pink colored solution was judged to be pure by polyacrylamide gel electrophoresis (Fig. 3). It can be seen that lactoferrin constitutes a major whey protein in monkey milk, as it does in human milk. In addition, migration of monkey and human lactoferrin in the polyacrylamide gels is identical.

Concentration of lactoferrin in several mature monkey and human milk samples was determined by rocket immunoelectrophoresis. The results indicate very similar lactoferrin concentrations in mature monkey and human milk with an average concentration in monkey milk of 1.73 mg/ml and in human milk of 1.82 mg/ml (Table 1).

Table 2. *Amino acid composition of monkey and human lactoferrin*

	Mol amino acid/mol protein	
	Monkey lactoferrin	Human lactoferrin*
Aspartate	72	67
Threonine	31	34
Serine	43	52
Histidine	9	12
Glutamate	67	68
Proline	34	36
Glycine	61	50
Alanine	73	61
Valine	50	41
Isoleucine	15	14
Leucine	62	53
Tyrosine	29	19
Phenylalanine	30	30
Lysine	47	44
Arginine	40	44
Methionine	1.2	3
Cysteine	23	26

* Data from Montreuil *et al.* (34).

Table 3. *Structure of N-terminus of human, monkey, and bovine lactoferrin*

	arg / \
Human Lf*	NH_3–gly–arg–arg–arg ser–val–gin–trp–cys–ala–val
Monkey Lf	NH_3–ala–arg–arg–arg–ser–val–arg–X^+–X–ala–val
Bovine Lf*	NH_3–ala–pro–arg–lys–asn–val–arg–trp–cys–thr–ile

* Data from Wang *et al.* (35).
† Not determined by amino acid sequencing.

Amino acid composition of monkey and human lactoferrin is compared in Table 2. The profile for the isolated monkey lactoferrin is very similar to previously published values for human lactoferrin (34). Human milk lactoferrin has been reported to have an unusual N-terminal, with four consecutive arginine residues (35). In contrast, bovine lactoferrin does not possess this highly basic N-terminal structure. Our analysis of monkey lactoferrin (Table 3) shows an amino acid sequence similar to that of human lactoferrin, with three consecutive arginine residues.

The content of the various monosaccharides in monkey lactoferrin as compared with human lactoferrin is given in Table 4. Total hexose and N-acetyl glucosamine content of the two glycoproteins is similar with monkey lactoferrin containing somewhat less galactose, fucose, and sialic acid and relatively more mannose.

Immunodiffusion was carried out to assess antigenic characteristics of lactoferrin from several species. This gives an indication of structural similarity of the lactoferrin molecules. With monkey lactoferrin antibody in the center well a precipitin band of identity was visible with human and monkey milk, signifying that the antibody could not distinguish between the human and the monkey protein. An additional band formed with the monkey lactoferrin, indicating further recognition of unknown determinants by the monkey antibody. No reaction occurred with milk samples from cow, goat, sheep, dog, or rat (Fig. 4). Identical results were obtained when antibody to human lactoferrin was placed in the center well.

Table 4. *Carbohydrate composition of monkey and human lactoferrin*

Monosaccharide	Mol monosaccharide/mol protein	
	Monkey lactoferrin	Human lactoferrin*
Galactose	2.2	4.2
Mannose	9.4	6.0
Fucose	0.8	2.2
Total hexoses	12.4	12.4
N-Acetyl glucosamine	8.2	8.3
N-Acetyl neuraminic acid	1.4	2.3

* Data from Legrand *et al.* (29).

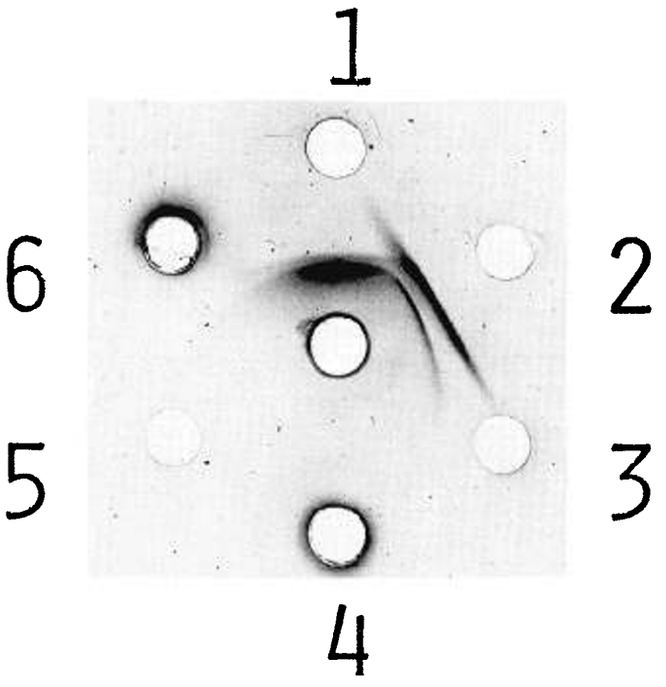

Fig. 4. Immunodiffusion of milk from various species against the antibody to monkey lactoferrin. *Center well*—monkey lactoferrin antibody; *1*, human milk; *2*, monkey milk; *3*, cow's milk; *4*, sheep milk; *5*, goat milk; *6*, dog milk. Rat milk not shown.

DISCUSSION

In the present study we have examined the characteristics of lactoferrin from monkey milk in order to assess the suitability of its use in the study of iron absorption from milk. Pure monkey lactoferrin was isolated by ion exchange chromatography followed by affinity chromatography on Heparin-Sepharose. As can be seen on the polyacrylamide gels (Fig. 3), lactoferrin comprises a major whey protein in monkey milk, as it does in human milk, compared to the minor band seen in cow's whey. Monkey milk is similar to human milk in its high content of lactoferrin as determined by immunoelectrophoresis. This is in contrast to most other mammals, whose milks contain significantly lower concentrations of lactoferrin, 10–100 times less than the amounts seen in human milk (19). Again in contrast to bovine lactoferrin, human and monkey lactoferrin have similar sequences at the N-terminal of the protein, each containing a highly basic terminal segment. Carbohydrate composition of the two glycoproteins is also similar. In studies examining the glycans of serum transferrins from several species and lactoferrins from human, bovine, and goat milks Spik *et al.* (36) have found fucose to be present only in human lactoferrin. Therefore, our finding of the presence of fucose in monkey lactoferrin is another demonstration of the similarity of human and monkey lactoferrin in contrast to other species.

Our data show the immunological characteristics of human and monkey lactoferrin to be very similar. A precipitation band, indicating immunological identity, formed between human and monkey milk, demonstrating a high degree of homology between these two proteins. No band developed with the other milk samples.

Lactoferrin has been investigated for its potential role as a vehicle for iron transport and absorption in the infant. An *in vitro* study by Cox *et al.* (17) demonstrated that of various iron-binding proteins (human lactoferrin, bovine lactoferrin, ovotransferrin, human serum transferrin) only human lactoferrin had the ability to deliver iron to the intestinal mucosa of normal adults. In a study with piglets it was shown that iron from lactoferrin was absorbed at a faster rate than iron as iron sulfate (37). Using iron-deficient weanling mice, lactoferrin iron was found to be bioavailable and to bring hematological parameters back to normal levels (38). These studies suggest a role for lactoferrin in facilitating iron transport across the brush border.

In a study with rats, Huebers *et al.* (39) concluded that lactoferrin inhibits iron absorption. Using intestinal loops they showed that human lactoferrin was poorly taken up by the mucosal cell. However, the rat does not appear to be a valid model for studying the role of lactoferrin in iron absorption. The major iron-binding protein in rat milk is transferrin, with lactoferrin being present in an extremely low concentration. In addition, the use of the nonhomologous system by Huebers et al. (39) (human lactoferrin in a rat system) is a limitation since significant species differences in lactoferrin structure and probably function may affect the results.

Significant evidence has accumulated regarding the mechanism of iron donation to cells by transferrin. The model, as proposed by Morgan (40) and supported by morphological (41) and isotopic data (42), proposes that transferrin binds to a specific membrane receptor, that the transferrin-receptor complex is internalized by endocytosis, that the iron is released inside the cell, and that the apotransferrin exits from the cell. This model has since been demonstrated in many cell types and species (43–46). A mechanism for iron donation similar to the model proposed for transferrin can be envisioned for lactoferrin in the brush border membrane and is supported in part by the finding of immunologically intact lactoferrin in the feces of breast fed infants (12, 13).

In summary, the mechanism of iron uptake into mucosal cells remains unclear. Data by Cox *et al.* (17) give evidence for specific receptors for lactoferrin in the small intestine. In addition, in a preliminary study we have recently shown specific binding of monkey milk lactoferrin to receptors in the brush border (47). Studies demonstrating high bioavailability of lactoferrin-iron and the fact that the protein seems to be protected against proteolysis in the gastrointestinal tract of the infant provide additional support for a role of lactoferrin in iron absorption. We have observed that the characteristics of monkey lactoferrin, as opposed to lactoferrin from other species, are quite similar to those of human lactoferrin. The concentration of lactoferrin in monkey milk is high, facilitating the extraction of relatively large amounts of the protein from monkey milk. Elucidation of the role of lactoferrin in iron absorption in the suckling infant and its role in inflammation and bacteriostasis may be possible using the monkey as an experimental model.

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